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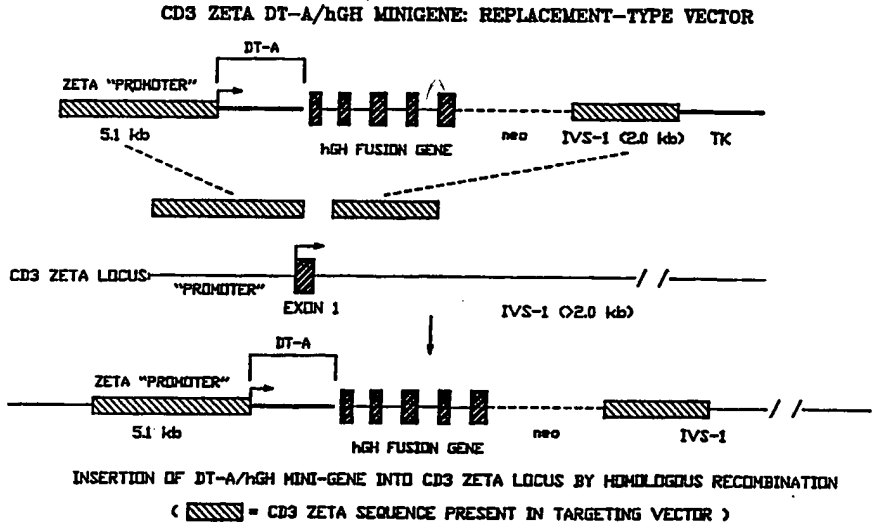

(57) Abstract

Transgenic non-human animals having genotypes comprising a first DNA sequence encoding an expression regulation sequence for a lymphoid gene operably linked to DNA encoding a lethal polypeptide. Also included are transgenic animals having genotypes comprising the substitution, deletion r insertion of one or more nucleotides in at least one endogenous allele f a CD3-type gene. The transgenic non-human animals have phenotypes characterized by immunodeficiency in at least one function f a lymphoid cell. Such phenotypes include depletion f ne or m re lymphoid cell types such as T-cells, NK cells, large granular lymphocytes, and/ r B-cells. The invention als provides transgenic immunodeficient animals containing a xenograft characterized by a phenotype wherein the animal has an enhanced ability to maintain the xenograft as compared to the maintenance of the xenograft in the species from which the transgenic animal is derived.

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TRANSGENIC IMMUNODEFICIENT NON-HUMAN ANIMALS

This is a continuation-in-part of application Serial No. 07/841,317, filed February 25, 1992, which is a continuation-in-part of application Serial No. 07/716,656, filed June 14, 1991.

FIELD OF THE INVENTION

The invention relates to transgenic non-human animals having phenotypes characterized by a substantial immunodeficiency in at least one function of a lymphoid cell such as a T lymphocyte, an NK cell, a large granular lymphocyte and/or a B lymphocyte. It also relates to transgenic immunodeficient animals containing xenographic cells or tissue.

BACKGROUND OF THE INVENTION

The vertebrate immune system can be functionally divided into cell compartments providing both adaptive immunity and innate immunity.

Innate immunity is characterized by a lack of specific recognition of particular foreign agents and provides the first line of defense against foreign pathogens, such as viruses, bacteria and protozoa. Cells of the

innate immune system, while unable to specifically recognize foreign pathogens, are adept at distinguishing normal, healthy host cells from infected, damaged or transformed host cells, and selectively killing these abnormal cells. In the case of infected cells, cell death is accompanied by destruction of the pathogen's refuge, and in the case of viral infections, often the interruption of nascent viral particle synthesis. For example, the ability of natural killer (NK) cells to efficiently distinguish healthy host "self" cells from infected or otherwise "nonself" cells and to effectively kill the latter accounts for the significant role these lymphoid cells play in tissue graft and transplant rejection.

Adaptive immunity is a complex defense system that is also able to recognize and kill invading organisms such as bacteria, viruses, and fungi, but reacts to foreign antigens with a cascade of molecular and cellular events that ultimately results in the humoral and cell-mediated immune response. This pathway of the immune defense generally commences with the trapping of the antigen by antigen presenting cells (APCs), such as dendritic cells and macrophages. These cells of capable of internalizing, partially digesting, and displaying the "processed" antigen on their cell surfaces. The adaptive immune response of the vertebrate system relies, in part, on cells of the lymphoid line. These cells include B cells, which give rise to soluble antibodies, and T cells, including T helper, T suppressor, and cytotoxic T cells.

T lymphocytes recognize processed antigen in the context of one of two classes of self Major Histocompatibility Complex (MHC) molecules, Class I and Class II. These proteins are the "antigen presenting" proteins found on the surface of APCs. Specific

recognition of the APC is accomplished by means of the T-cell antigen receptor (TCAR) and either a CD8 protein, specific for Class I MHC and expressed on the surface of cytotoxic (T8) T-cells, or a CD4 protein, specific for class II MHC and expressed on the surface of helper (T4) T-cells. Thus, two different types of T cells are involved in antigen recognition within the context of self MHC (major histocompatibility locus). Mature T helper cells (CD4+ CD8-) recognize antigen in the context of class II MHC molecules, whereas cytotoxic T cells (CD4- CD8+) recognize antigen in the context of class I MHC determinants.

The TCAR is noncovalently associated with CD3, itself a noncovalently associated complex of 5 invariant polypeptide chains; namely, γ, δ, ϵ, and either a $\zeta\zeta$ or a $\zeta\eta$ heterodimer (Allison, J.P., et al., (1987) Ann. Rev. Immunol. 5:503). The η chain displays substantial sequence homology to the ζ chain. The majority (80-95%) of CD3 complexes contain the $\zeta\zeta$ homodimer (Ashwell, et al. (1990) Ann. Rev. Immunol. 8:139-67). Similarly, NK cells display on their surface a receptor for the Fc portion of IgG, known as FcγRIII. CD3ζ has also been identified on NK cells and reportedly shown to specifically associate with FcγRIII (Lanier et al. (1989) Nature 342:803-805).

Naturally-occurring immunodeficient mice have been used to study the immune system, cancer, and infectious diseases, including acquired immune deficiency syndrome or AIDS. Several different strains with quite distinct immunodeficient phenotypes have been widely used in these studies. For example, the nude (NU) mouse is athymic, so T cell differentiation and maturation cannot occur. Nude mice have served for many years as host for xenografts, especially human tumors and the testing of anti-cancer drugs. The sever combin d

immunodeficiency syndrome (SCID) mouse appears to defectively rearrange both TCAR (T cell receptor) and immunoglobulin genes and displays a severe immunodeficiency. The beige (BG) mouse carries a defect in functional natural killer cells, whereas the X-linked immunodeficient (XID) mouse has a defect in the production of B cells. In addition, crosses have been made among various strains to generate lines with more comprehensive immunodeficient pheno-types (e.g., BG/NU and BG/NU/XID).

Unfortunately, available immunodeficient mouse strains do not tolerate all transplants. Neither do all graphs maintain the phenotypes observed in the original host. Despite the survival of most human tumors in nude mice, many are nevertheless subject to immune rejection.

A recent attempt to generate an animal model to study AIDS and bone marrow cell differentiation has been reported in which human lymphocytes are transiently proliferated upon coengrafting human fetal liver, thymus, and lymph nodes into SCID mice to form a SCID/hu mouse (McCune et al. (1988) Science 241:1632-1686). Human immune tissues in these mice are susceptible to human immunodeficiency virus (HIV) infection (Namikawa et al. (1988) Science 242:1684-1686) and the model has recently been used to test the effectiveness of AZT in delaying the replication of the AIDS virus. These mice, however are quite limited in their ability to sustain long term engraftment and support development of multiple human immune tissues. Furthermore, experiments in which either human peripheral blood lymphocytes (D. Mosier, et al. (1988) Nature 335:256-259), human bone marrow (S. Kamel-Reid, et al. (1988) Science 242:1706-1709) or human bone marrow plus neonatal thymus (Barry, T.S. et al. (1991) J. Exp. Med. 173:167-180) were engrafted into

immunodeficient mice have also led to partial transient reconstitution of human lymphocytes.

Because of the above limitations of naturally occurring in immunodeficient mice, investigators have attempted to experimentally generate immunodeficient mice by inactivating specific cell lineages or gene function during mouse development.

It has been reported that class II-specific CD4+ CD8- helper T cells (also referred to as T4 cells) fail to develop in mice neonatally treated with anti-class II MHC monoclonal antibody (Kruisbeek, A.N., et al. (1983) J. Exp. Med. 157:1932-1946; Kruisbeek, A.N., et al. (1985) J. Exp. Med. 161:1029-1047). Similarly, it has recently been reported that mice chronically treated with anti-class I MHC monoclonal antibody from birth have a significantly reduced population of CD4- CD8+ cells in cytotoxic T cell precursors (Marusic-Galesic, S., et al. (1988) Nature 333:180-183).

A transgenic mouse line has also been constructed containing a transgene encoding a rearranged β-chain of the TCAR wherein the variable region was deleted (Krimpenfort, et al. (1989) Nature 341:742-746). This resulted in a transgenic mouse depleted in mature splenic T-lymphocytes. See also PCT Publication WO 90/06359.

Transgenic mice reportedly have been produced containing herpes simplex virus thymidine kinase (HSV-TK) transgene fused to the Ig promotor/enhancer. Transgenic cells that express the HSV-TK, primarily B cells, initially developed unremarkably. However, upon administration of GANC, replicating B cells, expressing the HSV-TK gene, were reportedly killed (Heyman, et al. (1989) Proc. Natl. Acad. Sci. 86:2698-2702).

In another approach, mice carrying elastase/diphtheria toxin A (DT-A) fusion gene reportedly lacked a normal pancreas (Palmiter, et al. (1971) Cell 50:435-443). In addition, it has been reported that microphthalmia in transgenic mice resulted from the introduction of the DT-A gene fused to the α2-crystalline promoter (Breitman, et al. (1987) Science 238:1563).

The references discussed above are provided solely for the disclosure prior to the filing date of the present application and nothing herein is to be construed as an admission that the inventors are not entitled to antedate of such disclosures by virtue of prior invention or priority based upon earlier filed applications.

Given the state of the art, it is apparent that a need exists for an immunodeficient host that does not require continual antibody treatment and which is deficient in adaptive and/or innate immune responses and so provides better transplant recipients for the engraftment of foreign (xenographic) tissues.

Accordingly, it is an object herein to provide transgenic non-human animals having genotypes characterized by transgenes comprising DNA encoding a lethal polypeptide the expression of which is under the control of specified expression regulation sequences.

Further, it is an object herein to provide transgenic non-human animals having genotypes characterized by the substitution, deletion or insertion of one or more nucleotides in at least one endogenous allele of at least one CD3-type gene.

It is also an object herein to provide transgenic non-human animals having ph notypes characterized by a

substantial immunodeficiency in at least one function of a lymphoid cell such as a T lymphocyte, NK cell, large granular lymphocyte and/or B lymphocyte.

Further, it is an object herein to provide a transgenic non-human animal substantially depleted in functional T lymphocytes, NK cells, large granular lymphocytes and/or B lymphocytes.

Still further, it is an object herein to provide animal cells having genotypes characterized by a first DNA sequence encoding an expression regulation sequence for a lymphoid gene operably linked to a second DNA sequence encoding a lethal polypeptide.

It is also an object herein to provide animal cells having genotypes characterized by the substitution, deletion or insertion of one or more nucleotides in an endogenous allele of at least one CD3-type gene.

It is a further object of the invention to provide transgenic immunodeficient animals having a phenotype characterized by their enhanced ability to maintain xenographs and transgenic immunodeficient animals containing such xenographs.

Further, it is an object herein to provide transgenes capable of producing such transgenic non-human animals.

Still further, it is an object herein to provide methods for making and using such transgenic immunodeficient non-human animals.

SUMMARY OF THE INVENTION

The invention includes transgenic non-human animals having one of three different genotypes or combinations thereof. The first two genotypes are characterized by a first DNA sequence encoding an expression regulation sequence for a lymphoid gene operably linked to DNA encoding a lethal polypeptide. The third genotype is characterized by the substitution, deletion or insertion of one or more nucleotides in at least one endogenous allele of a CD3-type gene.

Transgenic non-human animals having Genotype I contain a transgene that is randomly integrated into the genome of the transgenic animal. The Genotype I transgenic non-human animal includes a first DNA sequence encoding an expression regulation sequence for a lymphoid gene operably linked to a second DNA sequence encoding a lethal polypeptide. In Genotype I transgenic non-human animals, the expression regulation sequence of the first DNA sequence incorporates sufficient control sequences such that it is capable of controlling temporal and tissue specific expression of the second DNA sequence encoding the lethal polypeptide.

Genotype II transgenic non-human animals are characterized by a DNA sequence encoding a lethal polypeptide that is integrated into the genome at a predetermine site such that its expression is controlled by an endogenous expression regulation sequence of a lymphoid gene. The transgenes used to make Genotype II animals are designed to target the integration of the transgene to the predetermined site by homologous recombination.

Genotype III transgenic animals are characterized by the substitution, deletion or insertion of one or more

nucleotides in an endogenous allele of at least one CD3-type gene. Genotype III animals are produced by use of a transgen designed for homologous recombination with an endogenous allele of CD3-type gene to encode the substitution, deletion or insertion of one or more nucleotides in the endogenous allele. The effect of such modification is the disruption of expression of a functional gene product by the recombined allele.

The invention also includes transgenic non-human animals having phenotypes characterized by immunodeficiency in at least one function of a lymphoid cell. Such phenotypes include depletion of one or more lymphoid cell types such as T-cells, NK cells, large granular lymphocytes, and/or B-cells. In addition, the transgenic immunodeficient animals of the invention can be crossed with other animals, either within the scope of the invention or otherwise to produce a transgenic immunodeficient animal having multiple desired phenotypes. For example, the transgenic immunodeficient mouse of the invention having a phenotype characterized by a substantial immunodeficiency in T and NK cells can be combined with a phenotype wherein B cell function is immunocompromised to produce an animal which is immunocompromised in T, NK and B cells.

The transgenic immunodeficient animals of the invention can be used to study the effects of a particular phenotype on innate and/or adaptive immunity. A specific and important utility of such animals, however, is their use as a host for transplantation of xenographs. Thus, the invention provides xenographic transgenic animals characterized by a phenotype wherein the animal has an enhanced ability to maintain xenographic cells and/or tissue as compared to the maintenance of the same xenographic cell and/or tissu

in the species from which the transgenic animal is derived.

DETAILED DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the intron-exon organization of the murine CD3ζ chain gene.

Fig. 2 depicts the BamHI restriction digest map of the murine CD3ζ chain gene.

Fig. 3 is the cDNA sequence encoding the murine CD3ζ polypeptide as reported by Baniyash, et al. (1989) J. Biol. Chem. 264:13252.

Fig. 4 depicts the structure of the CD3-T-cell antigen receptor (TCAR) complex.

Figs. 5A and 5B depict transgenes for inactivating CD3ζ genes. Specific embodiments include the insertion (Fig. 5A) and deletion (Fig. 5B) of one or more nucleotides (exons 3-7) of a CD3ζ gene.

Fig. 6 depicts an insertion-type vector wherein homologous recombination at a single cross-over point leads to the insertion of the targeting vector into the endogenous locus. The targeting vector is treated with a restriction endonuclease that linearizes the DNA within the CD3ζ chain sequence.

Fig. 7 depicts a transgene of the invention for CD3ζ inactivation comprising a positive/negative selection (PNS) vector.

Fig. 8 depicts a transgene encoding a CD3ζ expression regulation sequence operably linked to a second DNA

sequence encoding a lethal polypeptide designed for random integration into the genome of a transgenic non-human animal.

Figs. 9A and 9B depict transgenes encoding a lethal polypeptide for targeted homologous recombination with an endogenous CD3ζ gene. Fig. 9A is an insertion-type transgene cleaved by an endonuclease in the ζ promoter sequence before integration. Fig. 9B depicts a transgene encoding a lethal polypeptide designed for homologous replacement within the CD3ζ allele.

Figs. 10A through 10D depict the various plasmids used to construct pζDTneo.

Fig. 11 is a linear map of an endogenous CD3ζ gene and pζDTneo and the genomic structure obtained after a single crossover event between the endogenous CD3ζ allele and the transgene contained on pζDTneo.

Fig. 12 depicts the strategy for construction of transgenes for murine CD3ζ inactivation by homologous recombination.

Fig. 13 depicts hybridization probes for analyzing genomic DNA from ES cells for the presence of CD3ζ gene inactivation.

Fig. 14 depicts the strategy for construction of transgenes for murine CD3γ/δ inactivation by homologous recombination.

Fig. 15 shows a restiction map and probes for analyzing genomic DNA from ES cells for homologous integration of transgenes for CD3γ-δ inactivation.

Figs. 16A through 16I depict the FACS analysis of ES- and host-derived B and T cell populations in chimeras and control animals.

Fig. 17 shows experiments with mice homozygous for the inactivated CD3ζ allele. Fig. 17A epicts the structures of a targeting vector and a targeted CD3ζ allele. Fig. 17B shows that animals represented by lanes 8, 9, 10 and 11 have the homozygous genotype.

Fig. 18 depicts the examination of peripheral blood lymphocytes. Panel A shows the normal single staining profile for populations of B and T cells. Panel B shows a complete absence of CD3ε positive cells in the -/- CD3ζ K.O. mouse. Panels C and D show staining with anti-NK1.1 antibody indicating the presence of approximately 5-6% NK1.1 positive cells in two -/- mice.

Fig. 19A, spleen cells from +/+ and -/- mice stained for CD3ε vs. TCR α/β chains. Fig. 19B shows splenocytes stained with CD3ε vs. TCR γ/δ. Fig. 19C shows splenocytes stained for both TCR α/β and CD4 or CD8.

Fig. 20. Staining of thymocytes from mice as in Fig. 19. Fig. 20A shows that thymocytes from +/+ mice coexpressed TCRα/β and CD3E (12%) while those from two -/-mice did not. Figs. 20B and 20C show that even though the -/- thymocytes expressed relatively normal although lower amounts of CD4 and CD8, they never developed into CD3ε positive cells.

Fig. 21 depicts the strategy for constructing the CD3ζDT targeting vector and characterization of targeted ES cell clones.

Fig. 22 shows the FACS analysis of PBLs from founder DT077 and a C57BL/6 control.

Fig. 23 depicts the Hit-and-Run strategy for targeted DT expression.

DETAILED DESCRIPTION OF THE INVENTION

The invention includes transgenic and mosaic non-human animals having three defined genotypes characterized by three different types of transgenes incorporated into the genome of one or more cells of the transgenic or mosaic animal. The first two genotypes utilize DNA encoding a lethal polypeptide. The third genotype is characterized by the substitution, insertion or deletion of nucleotides into an endogenous allele to disrupt the expression of a functional gene product. The invention also includes xenographic transgenic animals having a phenotype characterized by the enhanced ability to maintain xenographic cells and/or tissue as compared to the animal from which the transgenic animal is derived.

Various aspects of the invention are common to each of the foregoing as set forth in the following definitions.

Definitions

The "non-human animals" of the invention comprise any non-human animal having an immune system which utilizes the genes or gene products specifically identified herein as well as structural and functional homologs thereto. Such non-human animals include vertebrates such as non-human primates and murine, rattus, ovine, canine, feline, bovine, porcine, equine species and the like. Preferred non-human animals are mammals

including rat, guinea pig, rabbit and mouse, most preferably mouse.

As used herein "animal cell" includes cell types derived from the above animals as well as from humans. A specific animal cell type is a "hematopoietic stem cell". Such stem cells give rise to the formed elements of the blood and other tissue which can be shown to be multipotent, to seed other organs and to renew themselves for the creation of further stem cells. Hematopoietic stem cells give rise to progenitor cells which in turn give rise to myeloid cells and lymphoid cells.

As used herein, "transgene" comprises nucleic acid (usually DNA) introduced into an embryonal target cell or integrated into the chromosome of the somatic and/or germ line cells of a non-human animal by way of human intervention, such as by way of the methods described herein. The particular embodiments of the transgenes of the invention are described in more detail hereinafter.

As used herein, "embryonal target cells" are cells into which the transgenes of the invention are introduced to produce the "transgenic non-human animals" of the invention. Examples include embryonic stem (ES) cells and the fertilized oocyte (zygote). In the case of zygotes the preferred method of transgene introduction is by microinjection. For ES cells, the preferred method is electroporation. However, other methods such as retroviral infection or other viral delivery systems, or liposomal fusion can be used.

"Lymphoid cells", as used herein, include at least T lymphocytes (T-cells), large granular lymphocytes (LGL), B lymphocytes (B-cells), and natural kill r (NK)

cells. T lymphocytes can be further divided into T-helper cells, cytotoxic T cells and T suppressor cells. Such cell types have recognized immune function and express cell surface markers well known in the art. For human surface markers see, e.g., Leucocyte Typing I, Bernard, A., et al., eds. (1984) Springer-Verlag to Leucocyte Typing II, Reinherz, E.L., et al., eds. (1986), Springer-Verlag; Leucocyte Typing III, McMichael, A.J., et al., eds. (1987), Oxford University Press; Leucocyte Typing IV, Knapp, W., et al., eds. (1990), Oxford University Press. In general, such human surface markers have homolog surface markers in analogous cell types in other species. For example, many of the surface markers characteristic of various cell types in human have well defined homologs in lymphocytes of other species. For references to mouse nomenclature, see Genetic Variants and Strains of the Laboratory Mouse, second edition, Lyon, M.F. and Searle, A.g., eds. (1989). Such homologs can be readily determined by those skilled in the art based upon cross-reactivity with monoclonal antibodies specific for the above identified markers, nucleic acid sequence homology based upon hybridization or direct comparison of nucleic acid sequence and homology as among amino acid sequence. Alternatively, or in conjunction with the foregoing, a homolog surface marker is determined by comparing the function of a particular marker.

As used herein, a "lymphoid gene" refers to a gene encoding a polypeptide involved in immune function of one or more lymphoid cells, or that are associated with proteins having known immune function in lymphoid cells including lymphoid cell specific developmental function. By way of example, the CD3ζ polypeptide is associated with the CD3 complex. The CD3 complex is associated with the TCAR on T lymphocytes. CD3ζ is

also associated with the FcγRIII receptor of NK cells. When associated with the TCAR, CD3ζ is involved in signal transduction. Thus, the gene encoding the CD3ζ polypeptide is a lymphoid gene. On the other hand, a gene encoding an enzyme in the biosynthetic pathway of all cells or a gene encoding a protein not involved in immune function is not a lymphoid gene. The expression of lymphoid genes, however, is not necessarily restricted to lymphoid cells. Thus, the gene encoding FcγRIII (also referred to as CD-16) is a lymphoid gene because it is expressed by NK cells. It is also expressed, however, by specific cells of myeloid origin, e.g., granulocytes.

As used herein, "operably linked" when describing the relationship between two DNA or polypeptide sequences means that they are functionally related to each other. For example, a promoter is operably linked to a coding sequence that controls the transcription of the sequence; a polyadenylation site is operably linked to a coding sequence if it is positioned so as to permit proper transcription termination and polyadenylation.

As used herein, "substantial immunodeficiency of at least one function" of a cell type refers to the modulation of an immune function associated with a particular cell type.

A. Genotypes Comprising DNA Encoding a Lethal Polypeptide

As indicated, the genotypes characterized by a DNA sequence encoding a lethal polypeptide comprises at least two distinct genotypes. Common to both genotypes, however, is the DNA sequence encoding the lethal polypeptide.

Such DNA sequences encode eith r a dominant lethal or a conditional lethal polyp ptide. A dominant lethal DNA sequence confers a dominant phenotype because expression results in the production of a polypeptide which is toxic to the cell producing it. Such dominant phenotypes are conferred by dominant lethal DNA sequences encoding proteins such as diphtheria toxin and ricin. An example of a conditional lethal DNA sequence which confers a conditional lethal phenotype is the Herpes Simplex Virus thymidine kinase gene (HSV-TK). This gene encodes an enzyme capable of converting a non-toxic substrate to a cytotoxin. The conditional aspect of this phenotype is based upon the requirement that the non-toxic substrate be supplied to the cell so that it is converted to a cytotoxin at the site of transgene expression.

In the broadest aspect of this embodiment of the invention, it is preferred that DNA encoding dominant lethal polypeptides be used in conjunction with an expression regulation sequence of a lymphoid gene. Use of such dominant lethal polypeptides provides assurance that the cell types expressing the lymphoid gene (from which the expression regulation sequence is derived or targeted) are ablated in the transgenic animal. The use of DNA encoding a conditional lethal polypeptide is less preferred because the ablation of cells does not occur until administration of a non-toxic substance that is converted by the gene product to the cytotoxin. The number and type of cells ablated in such instances depends upon the expression regulation sequence used, the type of conditional lethal polypeptide encoded by the DNA sequence and the timing and duration of administration of the non-toxic substrate. For example, the use of the HSV-TK gene in conjunction with gancyclovir ablates only those cells actively xpressing the TK gene when gancyclovir is administer d

and which are also actively replicating. While animals utilizing such conditional lethal genes may be useful to study an immune system of the animal, a finite probability exists for clonal escape. Further, difficulties may occur due to the inaccessibility of the target cells by the substrate, terminal differentiation of the cell type prior to administration of substrate, or the need to continuously administer the substrate for long periods. Thus, the use of conditional lethal polypeptides is not preferred when the animal is to be used for other purposes, such as a host for xenographs.

The DNA encoding the lethal polypeptide may be prokaryotic in origin and hence likely to be devoid of intervening sequences or introns. However, this embodiment of the invention requires the expression of such genes in eukaryotic cells which under natural conditions most often do not express genes without introns. Hence, in addition to regulatory sequences necessary to effect expression of the DNA encoding the lethal polypeptide, the transgenes of this embodiment of the invention may also encode DNA sequences that otherwise enhance the transcription or intracellular processing of the encoded lethal polypeptide. Such DNA sequences include eukaryotic genes or portions thereof which include intervening sequences or introns. An example of such a gene is human growth hormone (hGH).

1. Genotype I

The first genotype (Genotype I) is characterized by the incorporation of a transgene comprising first and second DNA sequences. The first DNA sequence encodes an expression regulation sequence from a specified gene which imparts tissue and temporal specific expression of the second DNA sequence. Such expression specificity is similar to that observed for the gen

from which the expression regulation sequences is obtained.

As used herein, "an expression regulation sequence" refers to DNA sequences associated with a lymphoid gene or specified lymphoid gene that are sufficient to confer tissue and temporal-specific expression of the DNA encoding the lethal polypeptides. Such sequences are generally located upstream from the translation initiation site for the lymphoid gene and are referred to as 5' flanking sequence. Such sequences include promoter sequences and in some instances enhancer sequences. In addition, 3' flanking sequences from the specified endogenous gene can be included in the transgene construct preferably positioned downstream from the second DNA sequence encoding a toxic polypeptide. The amount of 5' and optionally 3' flanking sequence can be readily determined by one skilled in the art by varying the amount of 5' or 3' flanking sequence included within a particular transgene construct and detecting in test transgenic animals, e.g., mice, the effect of varying the amounts of such sequence. Of course, in addition to those sequences required for tissue specificity of transcription, other sequences required for translation well known to those skilled in the art, e.g., polyadenylation sites, etc. are included in the Genotype I transgenes. Preferably, the expression regulation sequence comprises isogenic DNA from the same species and preferably same strain of non-human animal used to practice the invention. However, it is to be understood that expression regulation sequences from other strains or species of animal (including human), can be utilized in practicing the invention. All that is required is that such sequences provide sufficient specificity for transgene expression such

that tissue- and temporal-specific expression is obtained.

Since transgenes used to form Genotype I transgenic animals are designed for random integration, the preferred method for making transgenic animals utilizing this transgene is microinjection of the fertilized oocyte (zygote) of a non-human animal. Microinjection is a well-known method for forming transgenic non-human animals such as mice, sheep, cattle, rabbit and the like.

In the mouse, the male pronucleus reaches the size of approximately 20 micrometers in diameter which allows reproducible injection of 1-2 pl of DNA solution. The use of zygotes as a target for gene transfer has the advantage that, in most cases, the injected DNA is incorporated into the host genome before the first cleavage (Brinster, et al. (1985) Proc. Natl. Acad. Sci. USA 82:4438-4442). As a consequence, all cells of the transgenic non-human animal carry the incorporated transgene. This, in general, is reflected by the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells harbor the transgene.

Retroviral infection can also be used to randomly introduce transgenes encoding lethal genes into a non-human animal genome although this method is less preferred. For example, developing non-human embryos are cultured in vitro to the blastocyst stage which are then used as targets for retroviral infection (Jaenich, R. (1976) Proc. Natl. Acad. Sci. USA 73:1260-1264). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan, et al. (1986), Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Pr ss, Cold Spring

Harbor, NY). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner, et al. (1985) Proc. Natl. Acad. Sci. USA 82:6927-6931; Van der Putten, et al. (1985) Proc. Natl. Acad. Sci. USA 82:6148-6152). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, supra; Stewart, et al. (1987) EMBO J. 6:383-388).

Alternatively, viral infection is performed at a later stage. Virus or virus-producing cells are injected into the blastocoele (Jahner, D., et al. (1982) Nature 298:623-628). Most of the founders are mosaic for the transgene since incorporation occurs only in a subset of the cells which form the non-human animal. Further, the founder may contain various retroviral insertions of the transgene at different positions in the genome which generally segregate in the offspring. Additionally, it is also possible to introduce transgenes into the germ line, albeit with low efficiency, by intrauterine retroviral infection of the mid-gestation embryo (Jahner, D., et al. (1982) supra).

2. Genotype II

The second genotype (Genotype II) is made by using a transgene that targets a second DNA sequence encoding a toxic polypeptide to a specified endogenous allele of a gene within a cell or transgenic animal. Such transgenes are designed so that homologous recombination with the predetermined endogenous allele results in incorporation of the transgene in a position in the endogenous allele such that expression of the second DNA sequence is under control of the expression regulation sequence at the predetermined locus. The transgene used for such targeting generally comprises

a first DNA sequence having substantial homology to the specific predetermined endogenous allele. The first DNA sequence comprises first and second portions such that the second DNA sequence encoding the toxic polypeptide is disposed between them. The first DNA sequence is chosen such that the genotype formed upon homologous recombination causes tissue and temporal specific expression of the toxic polypeptide similar to that of the targeted endogenous allele. In general, such targeting transgenes further comprise positive and optionally negative selection markers to facilitate selection of successful transformants wherein the transgene is integrated by homologous recombination. As with the transgenes for Genotype I, the second DNA sequence can confer a dominant or conditional lethal phenotype, preferably a dominant lethal phenotype.

In the preferred embodiment, the first DNA sequence having homology to an endogenous allele of an animal is preferably isogenic DNA, i.e., DNA that is identical or substantially homologous (i.e., greater than 99% sequence homology) with the targeted sequence in the endogenous allele. The use of such isogenic sequences favors homologous recombination events and therefore an increase in the frequency of such recombination. Moreover, to the extent one of the portions of the first DNA sequence encodes all or part of an expression regulation sequence of the endogenous allele, the use of isogenic DNA provides for the reconstitution of an expression regulation sequence at the endogenous allele identical to that found in the wild-type animal.

Genotype II transgenic animals are preferred over Genotype I animals when such animals are used as hosts for xenographs. The basis for such preference lies in the site of integration of the DNA encoding the lethal polypeptide. In Genotype I, random integration of th

transgene can result in modulated tissue and/or temporal specificity. Targeting the lethal DNA sequence to an expression regulation sequence of an endogenous allele of a lymphoid gene, on the other hand, is less likely to produce modulation in lethal gene expression. This is especially true when dominant lethal genes are used in that unregulated expression of such a gene in non-targeted tissue during a critical developmental time period can have adverse impact on the development of the transgenic non-human animal.

3. Lymphoid Genes for Genotype I and Genotype II

The following identifies lymphoid genes that can be used in practicing the invention utilizing DNA encoding lethal polypeptides. It is to be understood that the identification of the various genes hereinafter refers to the use of expression regulation sequences from such genes to make transgenes to produce the Genotype I animals as well as DNA sequences used to construct transgenes for targeting the expression of DNA encoding a lethal polypeptide to an expression regulation sequence at an allele of the identified gene.

(a) Lymphoid Genes for Expression of Lethal Polypeptides

In this aspect of the invention, any lymphoid gene can be used in conjunction with DNA encoding a lethal polypeptide. Examples of such lymphoid genes include CD1, CD2, CD3-γ, CD3-δ, CD3-ϵ, CD3-ζ, CD3-η, CD4, CD5, CD7, CD8, CD19, CD20, CD38, CD40, CD45, CD72, CD76, $p56^{lck}$, IL-2Rβ chain, J11d (heat stable antigen), fyn, NK1, NK2, $Fc_{\epsilon}RI$-γ chain, IL-2R β-chain, αTCAR, βTCAR, γTCAR, δTCAR, $Fc_{\gamma}RIII$, RAG-1, RAG-2, Ig-β (B29), and IgM-α (MB-1) and genes associated with immunoglobulin isotypes Igμ, Igδ, Igγ, Igα, Igϵ; Igκ and Igλ.

(b) Lymphoid Genes Expressed by All T-Cells

In this aspect of the invention, any gene expressed by all T-cells is used in connection with the second DNA sequence encoding the toxic polypeptide.

The DNA sequences used to construct such transgenes are derived from any one of the group of genes which are expressed by all T lymphocytes. T lymphocytes are defined as belonging to a class of lymphocytes that express γ/δTCAR or that coexpress α/βTCAR and either CD4 or CD8. Such genes include CD1, CD2, CD3-γ, CD3-δ, CD3-ϵ, CD3-ζ, CD3-η, CD5, CD7, $p56^{lck}$, RAG-1, RAG-2, IL-2Rβ, J11d and fyn. These genes encode a corresponding lymphoid polypeptide which are known to be expressed by all T-cells.

Since Genotypes I and II confer a dominant or conditional lethal phenotype for those cells which express the transgene, the phenotype of a transgenic animal containing Genotype I or II when utilizing the appropriate DNA sequences from these genes (or other genes commonly expressed in T-cells) is the ablation of T-cells.

However, to the extent that other cell types express one or more of the above identified genes, ablation of such cell types is also expected. For example, RAG-1 and RAG-2 are expressed in all T-cells for rearrangement of genes contained in the T-cell antigen receptor repertoire. However, they are also expressed in B-cells for the purpose of rearrangement of the immunoglobulin repertoire. Thus, the use of RAG-1 or RAG-2 expression regulation sequences or the targeting of a lethal DNA sequence to these loci results not only in the ablation of T-cells but the ablation of mature B-cells (plasma cells) as well.

(c) Lymphoid Genes Expressed by NK Cells

As used herein, an "NK cell" is a lymphoid cell line associated with the innate immun response in vertebrates and in particular mammals. In mouse, the NK cell line is generally characterized as positive for CD2, CD3ζ, CD16 (FcγRIII), NK1, NK2 alleles, IL2 receptor β chain, and LFA1 markers, and negative for CD4, CD8 and TCAR.

In this aspect of the invention, sequences are derived from lymphoid genes that are expressed by NK cells. Such genes include those encoding CD2, CD3-ζ chain, $P56^{lck}$, fyn, NK1, NK2, $Fc_\epsilon RI$-γ chain and IL-2Rβ-chain polypeptides.

When one or more of the foregoing are used to direct expression of a lethal gene in a transgenic animal, at least the NK cell population of the transgenic animal is ablated. In some instances, other cell types are affected. For example, T cells also express CD2, CD3ζ, $P56^{lck}$ and IL-2Rβ-chain and are likewise ablated. Sequences from these genes are therefore useful in generating transgenic animals deficient in T and NK cells.

(d) Lymphoid Genes Expressed by Large Granular Lymphocytes

As used herein, a "large granular lymphocyte" is a lymphoid cell associated with the adaptive immune response in vertebrates and in mammals in particular. In mouse, the genes presently known to be expressed by large granular lymphocytes include those encode the α and β chains of the T cell antigen receptor, the NK1.1/1.2 surface marker, the Fc_γ RIII receptor and the Fc_ϵ RI-γ chain polypeptide (Rodewald, H-R, et al. (1992) Cell 69:139-150). When sequences from the α or β T-cell antigen receptor gene are used to produc

Genotypes I or II, the transgenic animal so-formed is ablated in large granular lymphocytes and the subset of T cells which express the α, β TCAR. In addition, when the Fcγ RIII gene is used, large granular lymphocytes, macrophages, neutrophils and mast cells are also ablated. In this regard, it should be noted that the above granulocytic cells and presumably mast cells are derived from granulocytic precursors which are of a myloid origin. Thus, the ablation of specified cells within the lymphoid line can also result in the ablation of cells of myeloid origin.

(e) Lymphoid Genes Expressed by B Cells

The genes expressed by B cells that can be used in practicing this aspect of the invention include RAG-1, RAG-2, Ig-β (B29), IgM-α (MB-1), J11d (heat stable antigen), CD19, CD20, CD38, CD40, CD45, CD72 and CD76 lymphoid genes and genes associated with the immunoglobulin isotypes Igμ, Igδ, Igγ, Igα, Igϵ, Igκ and Igλ.

In the case of CD38, this gene is expressed in plasma cells, pre-B cells, immature T cells and activated T cells. As a consequence, the use of DNA sequences from this gene to produce the Genotype I and Genotype II transgenic animals of the invention confers a phenotype wherein B and T cells are ablated.

(f) Ablation Using CD3-Type Genes

In the examples, CD3 genes from mice were used to construct transgenic immunodeficient mice. A CD3 gene refers to DNA encoding all or part of a polypeptide contained within the CD3 complex. The CD3 complex is non-covalently associated with T-cell antigen receptor (TCAR). Fig. 4 depicts the structure of the CD3 complex and identifies each of the polypeptides contained therein. As can be seen, the complex

comprises γ, δ, ε and ζζ chains or γ, δ, ε and ζη chains.

Since the ζ (zeta) chain is a part of the CD3 complex, the effect of utilizing transgenes encoding CD3ζ-type genes is the disruption of the function of the CD3 complex and as a consequence T-cell function. The CD3ζ gene is also expressed in NK cells and is involved in one or more functions of the NK cell. One NK function involves the noncovalent association of a CD3ζ polypeptide with one or more receptors located on the surface of the NK cell. In the case of mouse, the CD3ζ polypeptide has been shown to be associated in particular with at least the $Fc_{\gamma}RIII$ receptor (Ravetch, J.V., and Kinet, J-P. (1991) Annual Rev. Immunol. 9:457-492) which is also present on the surface of human NK cells (Lanier, et al. (1989), Nature 342:803-805). It is to be understood, however, that this CD3ζ polypeptide may also be associated with other cell components including other receptors on NK-type cells. As a consequence, when CD3ζ is used in conjunction with DNA encoding a lethal polypeptide T and NK cells are ablated.

The genomic organization of the CD3ζ gene for mouse is described in Baniyashn, et al. (1989) Biol. Chem. 264:13252-13257. A BamHI restriction map of the murine CD3 gene is shown in Fig. 2. The cDNA sequence of the mouse ζ-chain gene is shown in Fig. 3. However, other CD3ζ-type genes exist in other animal species. For example, the human CD3ζ gene has been cloned using the murine cDNA sequence as a probe. Substantial homology exists between the human and mouse ζ chain DNA sequences (Weissman, A. et al. (1988) Proc. Natl. Acad. Sci. USA 85:9709-9713). Given the divergence between the murine and human species and the similarity in CD3ζ chain DNA sequence, it is expected that many other

mammalian species contain one or more CD3ζ genes which can be readily identified by substantial homology to the above DNA sequences and by similarity in function of the CD3ζ gene product.

Recently, the CD3δ and CD3ε have been shown to be expressed in human fetal liver NK cells (Lanier, L.L. (1992) J. Exp. Med. 175(4):1055-66). Hence, ablation of cells expressing these subunits by a transgene encoding a lethal gene operable linked to CD3δ or CD3ε expression regulation sequences results in a combined T and NK depleted animal. The CD3γ subunit reportedly has only been identified in T cells.

B. CD3 Gene Inactivation

Genotype III gene inactivation is sometimes referred to as gene knock out. In general, a single round of homologous recombination which a specified gene results in the knock-out of only one of two or more possible alleles. Accordingly, the cell or transgenic animal containing a specified gene knock out generally does not express the desired phenotype until such time as homozygosity is established. Thus, transgenic animals heterozygous for the knockout of one allele are preferably inbred to produce the homozygous state.

In the case of Genotype III (knock out of one or all CD3 alleles, e.g., CD3γ, δ, ε, η and ζ) the phenotype is characterized by either a depletion in the number of mature cells normally expressing the knocked out gene (e.g., T-cells) or the presence of such cells in either normal or abnormal numbers wherein the absence of one or more functional CD3 gene products causes a modulation in at least one immune function of that cell type.

In this aspect of the invention, the transgenes of the invention include DNA sequences which ar capable of CD3 gene inactivation. Such transgenes are preferably constructed with r gions that are homologous to the CD3 gene to be fuctionally disrupted. However, they contain a substitution, deletion, or insertion of one or more nucleotides as compared with undisrupted alleles of the same CD3 gene naturally occurring in the species. Preferably, such transgenes are derived by deleting nucleotides from isogenic DNA encoding the functional CD3 gene. Although the resultant mutated DNA sequence is incapable of being transcribed and/or translated into a functional CD3 gene product, such transgenes retain sufficient sequence homology with the CD3 gene from which they are derived that the transgene is capable of homologous recombination with the endogenous CD3 gene. Such homologous recombination is preferably carried out in, for example, an embryonic stem (ES) target cell, to disrupt the expression of the targeted CD3 gene. See, e.g., Thomas, K.R. and Cappechi, M.R. (1987) Cell 51:503-512; Hasty, P., et al. (1991) Nature 350:243-246. Upon homologous recombination between the transgene and the endogenous CD3 gene, at least one allele of the CD3 gene is fuctionally disrupted. Such functional disruption may be by interference in initiation of transcription or translation, by premature termination of transcription or translation or by production of a non-functional CD3 protein. Such transgenes are preferably designed for replacement of the endogenous CD3 gene (see Figure 5a). Although insertional transgenes may also be used (see Figure 6b), replacement transgenes are preferred because they significantly reduce the likelihood of secondary recombination and reversion to the wild-type CD3 gene.

ES cells are obtained from pre-implantation embryos cultured in vitro that are fused with embryos after manipulation to incorporate the transgene used (Evans, M.J., et al. (1981) Nature 292:154-156; Bradley, M.O., et al. (1984) Nature 309:255-258; Gossler, et al. (1986) Proc. Natl. Acad. Sci. USA 83:9065-9069; and Robertson, et al. (1986) Nature 322:445-448). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retrovirus-mediated transduction. By selecting for homologous recombination in ES cells, individual clones containing an inactivated CD3 gene are identified. The selected ES cells are thereafter combined with blastocysts from a non-human animal. These ES cells then colonize the embryo and contribute to the germ line of the resulting chimeric animal. For review see Jaenisch, R. (1988) Science 240:1468-1474. Offspring of germline chimeras are bred to generate true transgenic non-human animals which may be bred to form transgenic animals homozygous for the CD3 gene inactivation.

One method of targeting mutations into non-selectable genes such as CD3 genes via homologous recombination and for selecting for such events is termed positive/negative selection (PNS) (Thomas, K.R. and Cappechi, M.R. (1987) Cell 51:503). This method involves the use of two selectable markers: one a positive selection marker such as the bacterial gene for neomycin resistance (Neor); the other a negative selection marker such as the herpes virus thymidine kinase (tk) gene. Neor confers resistance to the drug G-418, while herpes tk renders cells sensitive to the nucleoside analog gangcyclovir (GANC) or 1-(2-deoxy-2-fluoro-b-d-arabinofuranosyl)-5-iodouracil (FIAU). These two genes are incorporated into a transgene according to the scheme outlined in Figure 7.

As indicated in Figure 7 the inactivation transgene of the invention is generally flanked by regulatory sequences that allow for its independent transcription in ES cells with sequences homologous to the target gene of interest. Attached to one end of the fragment is a second independent expression unit capable of producing the polypeptide for herpes virus tk. Upon transfection, a portion of the ES cell incorporate the transgene by random integration or homologous recombination. All of these transformants express neor and can be selected for by adding G418 to the culture medium. A small percentage of total integrations occur by homologous recombination between the cognate endogenous gene and identical paired sequences in the DNA transgene. As a result, one copy of the targeted gene is disrupted by insertion of neor and concurrently the sequence for herpes tk is lost. Random integrants, which occur via the ends of the transgene, contain herpes tk and remain sensitive to GANC or FIAU. Therefore, selection, either sequentially or simultaneously with G418 and GANC enriches for transfected ES cells containing the transgene integrated into the genome by homologous recombination.

Other strategies that select for homologous recombination events but do not use PNS may also be used. For example, a promoter that is active in ES cells is operably linked to a positive selection gene such as the neor gene whose transcription unit lacks its own polyadenylation (poly-A) signal sequence. This expression unit is targeted to an exon of the cognate CD3 gene. Upon integration into the ES cell genome, the neor gene is transcribed independently, as above. Stable transcripts from the neor gene require the presence of a poly-A site downstream. Thus, by targeting the neor gene to a transcription unit,

homologous recombinants are linked to the poly-A site of the target gene. In addition, the "hit and run" strategy of Example 2 can be readily adapted for use in this aspect of the invention.

C. Combined Genotypes

A major aspect of the invention is the development of phenotypes immunodeficient in T, B, NK and/or LGL cell functions. Thus, the genotypes of different animals can be combined to obtain a specific multiple genotype. Such combinations of genotypes are between the transgenic animals of the invention or between a transgenic animal of the invention and a naturally-occuring immunodeficient animal (or transgenic immunodeficient animal not of the invention). The methods of genotype combination include cross-breeding or use of a zygote or ES cell from an animal for integration of an appropriate transgene.

For example, crossing animals expressing T-cell immunodeficiencies and animals expressing B-cell immunodeficiencies produce phenotypes immunodefficient in both T- and B-cell function. To this end, animal strains that lack one or more of these immune lineages can be combined to provide novel phenotypes. For example, the following transgenic mice have been generated by gene knockout.

Endogenous gene	Reference
RAG-1	Mombaerts, et al. (1992) Cell 68:869-877
RAG-2	Shinkai, et al. (1992) Cell 68:855-867
MHC Class 2	Cosgrove, et al. (1991) Cell 66:1051-1066; Grusby, M.J., et al. (1991) Science 253:1417-1420
IL2	Schorle, et al. (1991) Nature 352:621-624
β2-microglobulin	Correa, et al. (1992) Proc. Natl. Acad.Sci.USA. 89:653; Pereria, et al. (1992) Embo. J. 11:25-31; Koller, et al. (1990) Science 248:1247; Zijlstra, et al. (1990) Nature 344:742

Other transgenic immunodeficient mice include those having a deficiency in mature T-cells (Krimpenfort, et al. (1989) Nature 341:742-746) and those wherein endogenous immunoglobulin production by B-cells has been disrupted (see, e.g., PCT Publication WO92/03918 published March 19, 1992). Naturally-occurring immunodeficient animals include the nude, SCID, beige and X-linked immunodeficient mice.

One or more of the foregoing genotypes can be combined with one or more of the genotypes of the present invention to produce animals having modulated immune systems wherein specific cell types are affected. Exemplary are the crosses shown in the following table

Cross		Cell Type Affected: T	B	NK	Class I MHC
i.	(CD3ζ k.o. X RAG or Igμ k.o.)	-	-	-/+	normal
ii.	(β_2M k.o. X CD3ζ_{pro}DT or β_2M k.o. X CD3ζ k.o. X Igμ k.o.)	-	-	-/+	absent
iii.	(CD3ζ_{pro}DT X RAG or Igμ k.o.)	-	-	-	normal
iv.	(HLA tg X i, ii, iii)	-	-	-	+HLA

k.o. = Knockout of endogenous alleles
DT = Diphtheria toxin
tg = Transgenic
HLA = Human Class I or Class II genes

D. Xenographic Transgenic Immunodeficient Animals

In addition to the foregoing, the invention includes xenographic transgenic animals. Such transgenic animals are chimeric in that they contain one or more xenographic cell types, e.g., cells from a different species of animal or strain of animal such as human hematophoeitic stem cells, human peripheral blood lymphocytes (PBL), human bone marrow, fetal tissue, organs and the like. In addition to containing such xenographic cells and/or tissue, such animals are characterized by a phenotype based upon the ability of such a transgenic animal to maintain such xenographic tissue as compared to the maintenance of the same cell and/or tissue type in the same species of animal from which the transgenic animal is derived. Particularly useful genotypes for use as recipients of xenographic cells and/or tissue include those specifically set forth herein, i.e., Genotypes I, II and III.

Various protocols can be used to transplant xenographic cells and/or tissues to transgenic animals having the

genotyp and/or phenotype of the invention. Thus, human peripheral blood lymphocytes (hu-PBL) are transferred to such animals using protocols similar to that published for SCID mice (Mosier, et al. (1991) Science 251:791; Mosier, et al. (1990) J. Clin. Imunol. 10:185). Alternatively, protocols for tranplantation of human bone marrow to SCID mice can be used to generate xenographic transgenic animals containing bone marrow tissue. Kamel-Reid (1989) Science 246:1597. Further, human fetal tissue can also be transplanted into the transgenic immunodeficient mice of the invention to generate xenographic animals wherein the human immune system has been reconstituted. Protocols applicable for such applications include those published relating to the generation of SCID/hu mice (Kyoizumi, S., et al. (1992) Blood 79(7):1704-1711; Peault, B., et al. (1991) J. Exp. Med. 174(5):1283-1286; McCune, J.M., et al. (1988) Science 241:1632-1639).

Transgenic mice deficient in both T and NK cell function are superior hosts for xenographics including a variety of human tumors as well as non-transformed cells. First, elimination of T-cell function manifests an immunodeficient phenotype similar to those of the nude and SCID. However, since all cells contain the functional mutation, there is no clonal escape of T-cells as seen in both the nude and SCID strains. Second, a combined defect in both T and NK cells not only reduces the rate or frequency of tissue rejection, but also provides "physiological space" for the survival and differentiation of cells transfected from human bone marrow cells.

Other important advantage of the transgenic animals of the invention are their ability to model the role of human cells in tumor rejection, HIV infection and

adoptive cell therapy; to study the effect of the depletion of the humoral immune response, cell-mediated immune response or both. Further, such animals are useful as a model system to study AIDS and SCID syndromes involving the depletion of NK, T and/or NK and T-cell types.

E. Use of Transgenic Immunodeficient Cells

Cells from the transgenic immunodeficient animals of the invention are also useful. For example, human bone marrow plus transgenic immunodeficient murine bone marrow xenographic chimeras can be generated by co-injection of the two marrow types into lethally irradiated mice that are syngeneic with respect to the donor source of murine bone marrow. Protocols for this application are described in Lubin, I., et al. (1991) Science 252:427-431.

The following is presented by way of example and is not to be construed as a limitation on the scope of the invention. Further, all references referred to herein are expressly incorporated by reference.

EXAMPLE 1

Targeted Expression of Diphtheria Toxin
Inserted into the Murine CD3-ζ Chain Locus

A. Molecular Cloning of Murine
CD3-ζ Chain Genomic Sequences

Portions of ζ-chain cDNA, corresponding to the sequence of Yaniyash, et al. (1989) J. Biol. Chem. 264:13252, were used to screen for genomic sequences present in lambda phage libraries derived from either D3 embryonic stem cells or Balb/c liver DNA (Clontech). Separate cDNA fragments consisting of a portion of exon one (approximately 80 bp, from the 5'-end of the cDNA to the Asp718 site) or a fragment of approximately 200 bp (spanning most of exon 2, all of exon 3 and most of exon 4) were used to screen the D3 ES cell λ phage library, while a fragment containing most of exon 8 (approximately 640 bp from the NdeI site to the 3'-end of the cDNA) was used to screen the Balb/c liver library. Protocols were those described in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, NY (1982), and described briefly below.

The bacteriophage titre was first determined on Escherichia coli strain NM538 (Clontech).

Approximately 600,000 phages were than plated by adding the required amount of stock to NM538 bacterial cells and plated onto 150 mM agar plates. Nitrocellulose filters were used to transfer phage clones which were screened by standard DNA hybridization procedures (Maniatis, et al. supra) using probes described above. Isolation of phage DNA, restriction enzyme analysis, agarose gel electrophoresis, and Southern transfer of DNA from gel to nitrocellulose filter were all done according to standard procedures (Maniatis, et al.,

supra). Hybridization with probes was performed according to the same procedure as the screening conditions described above.

Both separate and overlapping clones were isolated from both DNA libraries that contained sequences that included exons 1-8 of the CD3ζ chain gene. These included BamHI fragments of 5.5 kb (isolated using exon 1 cDNA); contiguous 4.2 and 2.3 kb fragments (using a cDNA BamHI fragment of approximately 200 bp consisting of the majority of exon 2, all of exon 3, and most of exon 4); and contiguous 3.3 and 4.5 kb fragments (using exon 8 cDNA). Portions of the first four fragments were sequenced to verify the presence of the expected exon/intron borders or (in the case of the 5.5 kb fragment) for identity to the 5'-end of the cDNA. All BamHI fragments were the same size as reported by Baniyash et al. Biol. Chem., 264:13252-13257 (1989), with the only discrepancy being in the size of the fragment containing the latter portion of exon 4 and exons 5-7. In the Balb/c library, that BamHI fragment was only 3.3 (vs 3.9) kb in size even though its 5'-end contained precisely 25 nt of exon four, beginning with the BamHI site, predicted by the published results of Baniyash, et al. supra. These BamHI fragments were subcloned into the BamHI site of pUC18 and used to construct vectors for either the heterologous expression of diphtheria toxin or for "knock out" homologous recombination vectors designed to inactivate the expression of the endogenous CD3ζ chain gene.

B. Construction of a Diphtheria Toxin Expression Vector for Targeting of DT Expression to the CD3ζ Chain Locus

The 5.4 kb BamHI fragment containing the first exon of CD3-ζ was modified for the expression of diphtheria toxin-A chain. An Asp718 site unique to this fragment

lies within the untranslated leader of the the CD3-ζ transcript but upstream of the start of translation. See Fig. 2. Sequencing of this genomic fragment showed 100% homology to the cDNA sequence beginning 16 bp upstream of, and continuing 62 bp 5', of the Asp718 site. The lambda clone containing this 5.4 kb BamHI fragment was digested with BamHI and Asp718. The resulting fragment, containing approximately 5.1 kb upstream of the Asp718 site, was separated by agarose gel electrophoresis and purified using Gene Clean II. It was then ligated into pMH2 which was derivd from pUC18 by insertion of a synthetic polylinker into the EcoRI and HindIII sites in this plasmid. The polylinker encoded the restriction sites shown in Fig. 10A. The fragment was ligated between the BglII and Asp718 sites present in the synthetic polylinker region. As a result, the BamHI and BglII sites were destroyed. The ligation reaction was used to transform competent E. coli strains JM109 or DH5α by standard methods (Maniatis, et al., supra). It was found that a recA-strain was required to avoid the loss of approximately 500 bp of sequence in a region approximately one kb upstream of the Asp718 site. The preferred method for obtaining unrearranged DNA of this 5.1 kb CD3ζ fragments is to plate bacteria from the initial transformation after heat shock onto agar plates followed by incubation at room temperature for 48 hours. Alternatively, the fragment is subcloned into a low copy plasmid such as pBR322. For large scale DNA preparation, the bacterial colonies obtained from growth at room temperature at 48 hours are used to incubate both cultures that are shaken at 37°C for preferably not more than 8 hours prior to lysis. The thus formed plasmid was designated p70-2 as shown in Fig. 10C.

The Asp718 site was chosen for the site of fusion between the untranslated leader of the CD3-ζ and a DT-human growth hormone (hGH) fusion sequence. Successful induction of tissue-specific expression of DT-A chain in transgenic mice has been accomplished using such a gene fusion strategy (Palmiter, R.D. et al. (1987) Cell 50:435-443; Behringer, R.R. et al. (1988) Genes & Development 2:453-461). In principle, the fusion of the coding region of the bacteriophage encoded DT gene to the genomic gene for human growth hormone creates a gene construct that resembles the intron/exon structure of a mammalian gene. Empirically, such genes function more appropriately in transgenic mice than comparable constructs that lack any intervening sequences (Brinster, R.L. et al. (1988) Proc. Natl. Acad. Sci. 85:836-840; Palmiter, R.D. et al. (1990) Proc. Natl. Acad. Sci. 88:478-482; Choi, T. et al. (1991) Mol. Cell. Ciol. 11(6):3070-3074).

DT A-chain and hGH containing plasmids were obtained from R. Palmiter (U. Washington, Seattle). The DT-A sequence was removed from pUC-DT-A (Palmiter, et al. (1987) Cell 50:435-443) as a EcoRI-DraI fragment and subcloned into the EcoRI and HindII sites of pUC (pUCDTAΔs). DT was excised from a pUC-DTΔs (Fig. 10A) by first cleaving with HindIII and filling in with Klenow. It was then cut with Asp718. The hGH sequence in pUChGH is derived from a genomic EcoRI fragment subcloned into pUC (Palmiter, et al. (1987) Cell 50:435-443; Palmiter, et al. (1983) Science 222:809-814). The hGH sequence was isolated from pUC-hGH by first digesting with BamHI (located in exon one) and filling in with Klenow, followed by excision with EcoRI. Plasmid pMH2 was digested with Asp718 and EcoRI. All three fragments were gel purified, ligated, and transformed as above to form pMH2DT-hGH as shown in Fig. 10A.

The components of the final vector consist of: (1) approximately 5.1 kb of CD3ζ genomic sequence including a portion of the untranslated leader and adjacent upstream sequence; (2) the DT-hGH fusion cassette from pMH2DT-hGH; and (3) a positive selectable marker for neomycin resistance (PGKneo), all linked contiguously and subcloned in a pUC18-based plasmid (Fig. 10D). The PGKneo sequence containing the PGK promoter, PGK polyadenylation sequence, neomycin resistance gene (pGEM7KJ1 (SalI)) was subcloned into pMH2DT-hGH as an EcoRI to SalI fragment into the same sites to form pDTneo as shown in Fig. 10B. Next, the 5.1 kb CD3ζ fragment in p70-2 (Fig. 10C) was subcloned as an SfiI to Asp718 fragment into pMH2-DT/neo at the same sites to produce pζDTneo (Fig. 10D). The final ligation of the CD3ζ fragment required transformation into a recA-strain, preferable DH5α, in order to prevent loss of CD3ζ sequence. In addition, the preparation of large scale plasmid DNA (e.g. for electroporation into ES cells) was performed immediately after overnight growth of the transformed cells to avoid stationary phase growth of bacteria. A colony from the transformation was picked and placed into a 3 ml broth culture of LB + 80 μg/ml carbanicillin and incubated at 37°C for approximately 8-10 hours. This culture was diluted 1:200 with a larger volume of LB or TB plus carbanicillin for overnight growth at 37°C with shaking. Alternatively, a bacterial colony from the transformation was added directly to broth media and shaken at 37°C for preferably not more than 8 hours. DNA was isolated using the alkaline lysis method (Maniatis, et al., supra.).

C. Isolation of ES Cells Containing Targeted Insertion of DT

The plasmid pζDTneo (Fig. 10D) contains a unique Apa I site located approximately 2.5 kb upstream of the Asp718 site approximately in the center of the 5.1 kb CD3ζ fragment. Approximately 25 μg of pζDTneo was linearized by digestion with 125 units of Apa I (Boehringer Mannheim) in 150 μl buffer (supplied by manufacturer) for between 6-14 hours at 37°C. The DNA was extracted with a 50:50 ratio of saturated phenol and chloroform followed by 100% chloroform.

A 10% volume of 3M NaOAc was added, plus 2.5 volumes of 100% ethanol and the DNA precipitated in dry ice 10 minutes or at 20°C overnight. The DNA was pelleted in an eppendorf microfuge for 10 minutes, rinsed once with 70% ethanol, and air dried in a NuAire biosafety cabinet under sterile laminar flow conditions. The DNA pellet was resuspended in 0.1X TE (1 mM Tris.HCl pH 7.5, 0.1 mM EDTA) and used for electroporation of ES cells. The ends of this linearized plasmid have CD3ζ promoter sequences which are homologous to the corresponding sequence in the endogenous CD3ζ gene. The orientation of these end sequences target this DNA for insertion-type homologous recombination into the CD3ζ gene.

1. Propogation of ES Cells

The AB1 line, derived from mouse strain 129, was obtained and maintained by methods described in Robertson, E.J., 1987, Teratocarcinomas and embryonic stem cells, IRL Press; Zijlstra, J., et al. (1989), Nature, 342:435-438. Briefly, ES cells are grown on STO cell feeder layers (Martin, G.R., et al. (1975), Proc. Natl. Acad. Sci. USA, 72:1441-1445) in DMEM medium supplemented with 15% fetal calf serum (FCS), 1 X non-essential amino acids (Gibco) and 10^{-4} M β-

mercaptoethanol, and 1000 units/ml myeloid leukaemia inhibitory factor (LIF) (Williams, R.L. et al. (1988) Nature 336:684-687) (AMrad Corp., Ltd., Kew Victoria, Australia). The ES cells are transferred approximateIy every three days and used for DNA transfections as described below.

2. Electroporation and Clonal Isolation

Methods for electroporating ES cells have been described (Thomas, K.R., and M.R. Capecchi, 1987, Cell 51:503-512). Rapidly growing ES cells are trypsinized and resuspended in ES cell media (above). After counting, the cells are pelleted and resuspended in Dulbecco's calcium and magnesium free phosphate buffered saline (GIBCO). Approximately 1 x 10^7 ES cells are mixed with 20 μg/ml of linearized DNA and exposed to a single, 250 V/cm 500 MF pulse at room temperature using a Bio-Rad electroporation apparatus. After 10 minutes, between 1 and 5 x 10^6 the cells are plated onto 100 mm dish containing STO cell feeders. After 24 hours, 400 μg/ml G418 is added until clones arise (approximately 8-10 days). Individual clones are transferred to 24 well plates and expanded to 2 x 10^6 cells. A portion are used to extract DNA for either PCR or Southern blot analysis. To isolate cellular DNA, the growth media is removed by aspiration and the cells rinsed once with phosphate buffered saline (PBS) (Maniatis, et al., supra.). Approximately 500 ul of digest buffer (0.1M EDTA; 50 mM Tris-HCl, pH 8.0; 0.5% SDS 1 mg/ml proteinase K) is added to each well (or dish) of cells. After moderate shaking to loosen the cells, the mixture is transferred to a 1.5 ml eppendorf tube and incubated at 50°C overnight. The mixture is extracted once with saturated phenol:chloroform (1:1) and centrifuged for 5 minutes in a microcentrifuge. The aqueous phase is removed and the DNA precipitated by adding two volumes of 95% thanol and shaking well.

The DNA is pelleted for 5 minutes in a microfuge, rinsed once with 70% ethanol, and dried in a Savant speed-vac. The DNA is resuspended in TE.

D. Characterization of Homolgous Recombination of pζDTneo into the CD3ζ Locus of Murine ES Cell Clones

Fig. 11 depicts the linear DNA maps for the endogenous CD3ζ locus and pζDTneo upon targeted integration into the CD3ζ gene. Note that in the process of constructing pζDTneo the BamHI site at the 5'-end of the 5.1 kb CD3ζ fragment in pζDTneo was eliminated and that a BamHI site was added at the junction of the 5.1 CD3ζ fragment and DT. In order to identify such a homologous recombination event, cellular DNA from approximately 5 x 10^6 cells was isolated for Southern blot analysis as above.

Approximately 10 μg of genomic DNA from parental ES cells as well as individual or pooled clones was digested with 75 units of BamHI (NEB) overnight at 37°C. The DNA was electrophoresed directly into 0.9% agarose gels and transferred onto Genescreen (DuPont) nylon membranes using 1X TBE or 10X SSC (Maniatis). The DNA was cross-linked to the filters using a Stratagene UV crosslinker. The filters were prehybridized in 0.5X nylon wash (1X = 14% SDS, 130 mM Na_2HPO_4, 14 mM Na_4EDTA, 0.2% Triton-X, titrated to pH 7.2 with H_3PO_4) for greater than one hour at 65°C. An approximately 350 bp genomic probe fragment (designated ζ350) was prepared by digesting the 5.4 kb BamHI fragment of CD3ζ present in pUc18 with Asp817 and BamHI followed by gel purification. The probe was radiolabeled with ^{32}P-dCTP using a nick translation kit (Boehringer Mannheim) and approximately 50ng of gel purified DNA. The filters were hybridized in 0.5X NW at 65°C overnight with approximately 2x10^6 CPM/ml probe

DNA. The filters were then washed succesively with 0.5X, 0.2X, 0.1X NW at 65°C for at least 30 minutes each.

Homologous recombination of pζDTneo into one allele of CD3ζ leads to two cellular BamHI fragments capable of cross-hybridizing with the ζ350 probe: (1) the endogenous 5.4 kb BamHI fragment, and (2) a novel 8.6 kb band derived from a BamHI site within the PGKneo sequence and an endogenous BamHI fragment at its 3'-end acquired as a result of the homologous integration (Fig. 21C).

Homologous recombinant clones were first identified using the above ζ350 probe to detect the appearance of the novel 8.6 kb BamHI fragment. The blot was stripped and reprobed with a 600 bp Pst I fragment (isolated from pUC-DT) spanning the DT gene. This probe hybridized to a 4.0 kb BamHI fragment defined by the BamHI site at the 5'-end of the DT gene and the BamHI fragment in the PGKneo sequence (Fig. 11) thus verifying the presence of an insert that includes DT, hGH, and PGKneo.

Verification of intact DT sequence was performed by DNA polymerase chain amplification (PCR) of the coding region of DT from homologous recombinant ES cell clones. Two PCR primers were synthesized. A 26mer with 100% homology to the untranslated region of CD3-ζ of the Asp718 site was used to prime from the 5'-end of the DT sequence. Its sequence is 5' - CAT CAG CGC CTC CTT TTC TCC TCA T - 3'.

A 24mer with 100% homology to a region of exon one of hGH just downstream of the fusion junction with DT was used to prime from the 3'-end of the DT sequence. Its

sequence is 5'- AGC TGT CCA CAG GAC CCT GAG TGG TT - 3'.

Approximately 1μg of genomic ES cell clone DNA was added to a PCR reaction mixture and amplified for 30 cycles in a Perkin Elmer Thermocycler according to manufacturer's specifications (directions for PCR were those provided by Cetus with reagent kit). One tenth of the reaction (10μl) was electrophoresed in a 1.0% agarose gel to determine the size of PCR amplified products. As a standard for comparison, an identical reaction, using approximately 30 pg of the vector pζDTneo was also performed. The ES cell clone DNA yielded the same sized band (approximately 750 bp) as the control test plasmid, pζDTneo. A portion of the PCR amplified DNA was then digested with BglII (at the 5'-end of DT) and SphI (at the 3'-end of DT), gel purified as above, and subcloned into either pUC 18 or 19 or M13 mp18 or mp19 for DNA sequencing. Sequencing reactions were performed using Sequencase 2.0 from U.S. Biochemical, Inc. The entire coding region of DT was thus analyzed to determine that no mutations or rearrangements had occurred as a result of insertion into the genome.

E. Blastocyst Injection of ES Cell Clones Containing Targeted Insertion of DT to the CD3ζ Promoter Region: Generation of Founder Chimeras

1. Microinjection of Embryos

ES cells containing the transgene of Section D, above, are introduced into fertilized embryos essentially as described by A. Bradley, "Production and Analysis of Chimeric mice", in Teratocarcinomas and embryonic stem cells, a practical approach, E.J. Robertson, eds., IRL Press, 1987.

2. Production of Transgenic Mice

Individual positive clones generated by above methods were injected into C57BL/6J blastulas and transferred to uteri of pseudo-pregnant recipients (Bradley, A., supra.) The presence of ES cell contribution to the chimera is detected by the appearance of the dominant agouti coat color contributed by the ES cells on the black background of C57BL/6J mice. G_0 males are bred with C57BL/6J females to identify founders with germ line transmission of the transgene. "Pseudomales" derived from sex conversion of a female host blastocyst by the male ES cell line are expected to transmit the transgene to 50% of their offspring. Presence of the transgene is confirmed by Southern blot analysis of tail DNA samples. Generally, cells expressing the transgene are non-viable. Breeding of G1 transgenics yields offspring homozygous for the mutation.

G_0 founder mice that show agouti coat color chimerism were analyzed for the presence of the targeted DT gene sequence as described in Example ID, above. Tail tip DNA i s prepared by and lysed as described for ES cell DNA, above.

3. Special Care in Handling of Immunodeficient Mice

Since transgenic animals derived from dominant negative mutation (expressing DT) (see Examples 1-2) or recessive insertional mutations (see Examples 3-4) are expected to be severely immunodeficient, strict precautions are taken to avoid infections. General guidelines for the care of immunodeficient animals are described (National Research Council, 1989, Chapter 4 in "Immunodeficient Rodents, A Guide to Their Immunobiology, Husbandry, and Use" (1989, National Academy Press).

4. Specific Pathogen Free Areas

All mice are kept in specific path gen free (SPF) areas are in Lab Product isolator cages (each receiving HEPA filtered air and separate from each other). All bedding, feed, and water are autoclaved prior to entering the room and are added to sterilized, reusable filter capped cages in a lamlnar flow biosafety cabinet. When possible all manipulations of the mice are performed inside the biosafety cabinet. Personnel working with these mice wear foot covers, lab coats, gloves, and hair bonnets.

5. Quarantine procedures

As a precaution against introducing pathogens, all incoming mice are certified by the supplier as germ-free, specific-pathogen free, defined-flora, and viral-antibody-free animals. Upon arrival these animals are quarantined in a separate room that has negative air pressure with respect to the connecting corridor. Any sick animals are transferred to the quarantine room and subject to bacteriologic and viral screening.

F. Analysis of Chimeras Containing Targeted Insertion of DT to CD3ζ

Preliminary analysis for the tissue specific expression of DT was made by analyzing blood from six founder chimeras derived from one targeted ES cell clone 147. The source of the ES cell line was from mouse strain AB1. The source of the recipient embryo for the AB1/ES cell was mouse strain C57/BL/6. The construct used was that shown in Figure 9A.

It is expected that regulated expression of DT by the CD3ζ promoter will affect only cells that developmentally express CD3ζ. This would include mature cells of the T and NK cell lineages, as well as

cells that differentiate from a common hematopoietic stem cell precursor that expresses CD3ζ.

Relative contribution of ES cell vs. host cell-derived tissues was determined by staining for glucose phosphate isomerase isozymes (GPI) (Bradley, A., in Teratocarcinomas and Embryonic Stem Cells, ed. by E.J. Robertson. IRL Press, 1987). Since regulated expression of the DT construct should allow for the normal development of non-DT-expressing tissues, detection of the ES cell GPI isozyme should reflect the overall degree of chimerism for a given animal. GPI levels were used to measure the contribution of the ES cells to red blood cells (RBC). In a separate assay, differential antibody staining was used to evaluate ES contribution to T and B cells from the same blood samples.

1. GPI Isozyme Analysis

ES cells from strain AB1 contain the GPI-a isozyme. The host strain C57/BL/6, however, contains the GPI-b isozyme. These isozymes can be separated by electrophoresis in cellulose acetate plates and stained as described by Bradley, supra. Blood samples were prepared from orbital eye bleeds. For GPI analysis, approximately 150ul whole blood was added to 1 ml of phosphate buffered saline (PBS) on ice. The cells were centrifuged at low speed in an E&K microcentrifuge for 2 minutes, the supernatant removed, and the cells frozen in dry ice. The samples were thawed and diluted with distilled water for application to cellulose acetate plates in the standard assay. The results are shown in TABLE I.

TABLE I

GPI Analysis

Sample animals	% contribution by host	% contribution by ES cells
1. C57/BL/6	100	0
2. 129	100	0
3. 80% zeta k.o. chimera	20	80
4. DT chimera 061	~95	3 - 5
5. DT chimera 062	~95	3 - 5
6. DT chimera 063	~90	5 - 10
7. DT chimera 065	65	35
8. DT chimera 066	~90	5 - 10
9. DT chimera 067	~90	5 - 10

C57/BL/6 mice were the source of the GPI-b isozyme and 129 mice were the source of GPI-a. The 80% CD3ζ chain knock-out chimera (described in Section E, Example 3, above) served as a control for normal ES cell contribution to the blood of a high percent coat color chimera. Although it is possible that the disruption of one of the zeta chain alleles could be detrimental to normal hematopoiesis, defects in RBC or lymphocyte production were not observed.

The GPI results indicated that all six DT-chimeras show some level of ES cell contribution to the red blood cell (RBC) compartment ranging from a few percent to 35%. Thus, incorporation of the targeted DT-vector into chimeric tissues does not prevent the differentiation of the RBC lineage, suggesting that offspring carrying this allele will be able to produce functional RBC. However, the overall levels of ES cell GPI in the blood of the high percent coat color DT chimeras (samples 4-9) were significantly lower than for the non-DT chimera (sample 3).

2. Lymphocyte Analysis

ES cell-derived T and B cells can be distinguished from host cells using differential fluorescent antibody staining. The four cell types can be separated by double staining with antibodies raised against I-A^b (both 129 and C57/BL/6 are of the H-2^b haplotype) and the 129 strain specific T and B cell antigen, Ly9. A portion of the blood samples obtained for GPI analysis, above, was removed for staining. Samples were stained first with a mixture of biotinylated mouse (H-2^d)-anti-mouse IA^b antibody (PharMingen) (2.5 ug/ml) and a rat anti-Ly9 antibody (gift of J. Ledbetter) (.05 µg/ml). They were then treated with a FITC-conjugated mouse anti-rat antibody (Boehringer Mannheim) followed by streptavidin-phycoerythrin (PharMingen). This resulted in the following staining pattern with C57/BL/6 and 129-derived T and B cells separating into four quadrants:

BL/6 B cells	129 B cells
BL/6 T cells	129 T Cells

I-A^b (PE)

Ly9 (FITC)

The results of this FACS analysis are shown in Figures 16A-16I and are summarized in Table II. These results indicate that the high percent coat color control

chimera (sample 3) produced T and B cells of both ES cell (65% of total lymphocytes) and BL/6 host origin (35% of total lymphocytes) (Fig. 16C). The ES cell T to B cell ratio was approximately 2:1. Of the six DT chimeras, only one (sample 7, DT065) produced as many as 6% total lymphocytes of ES cell origin (Fig. 16G). These had a T to B cell ratio of 1:2, the reverse of normally developing lymphocytes of this age and strain, and by GPI analysis contributed approximately 35% to the RBC population.

TABLE II

FACS Analysis of Chimeric and Control T and B cell Contributions

Sample	FACS ID# #11:/5	BL/6 % T cells	BL/6 % B cells	ES % T cells	ES % B cells
1. C57/BL6	009	48	52	0	0
2. 129	010	2	3	67	28
3. 80% chimera	011	21	14	41	24
4. DT061	013	52	45	1	2
5. DT062	014	52	44	2	3
6. DT063	015				
7. DT065	016	50	44	2	4
8. DT066	017				
9. DT067	018	72	25	1	3

A significant result is the ability of BL/6 bone marrow stem cells from the 80% control chimera (sample 3) to contribute up to 35% of its lymphocytes (Fig. 16C), but at the same time provide for only about 15% of its red blood cells (by GPI analysis). This suggests that even though there is a general relationship between the amount of GPI isozyme in the blood (RBC) and the degree of lymphocyte differentiation from a particular stem cell type, one can expect that under normal conditions a minority of bone marrow stem cells can significantly contribute to the expansion of the lymphocyte or

erythrocyte lineages. In other words, blood from the 80% chimera contained predominantly ES cell derived RBCs (80:20, ES:BL/6) but a relatively higher proportion of BL/6 derived lymphocytes (35:65, BL/6:ES). However, in examining the six DT chimeras (Fig. 16D-16I), none shows a relative expansion of ES cell derived lymphocytes to the same extent as BL/6 derived lymphocytes in sample 3. Even when present at 6% of total lymphocytes, the T to B ratio is reverse that of animals for this age and strain (e.g., sample 7, Fig. 16G). These results strongly suggest that DT expression reduces the number and ratio of T and B cells in the chimeric animals.

Three males derived from ES cell clone, 121 (isolated at the same time as clone 147), have given birth to a total of 4 agouti pups (1-3 weeks of age). FACS analysis of PBLs from founder DT077 is shown in Fig. 22. Its chimeric lymphocyte staining profile is identical to that of DT065, i.e., T and B cells are primarily of BL/6 origin, with 2% of B cells of ES cell origin. Tail blot analysis of the pups will confirm germline transmission of the targeted allele.

A male chimera generated from DT ES cell clone 82 has also produced a litter of 7 pups, all of which are agouti. Unless inactivated by mutation or gene rearrangement, offspring carrying the targeted DT allele are devoid of T and NK cells and have a significant decrease in the number of mature B cells.

G. Generation of Germline Chimeras Containing Targeted Insertion of DT to the CD3ζ Promoter Region

G_0 male founders are bred with C57Bl/6 females to derive germline transmission of the introduced DT construct. F_1 offspring displaying 100% agouti skin

color indicate successful transmission of the ES cell derived genotype. F_1 offspring are annalyzed for the presence of the transgene by Southern blot analysis of tail DNA (Fig. 21 and above). Male and female siblings from this cross are bred to derive F_2 generation mice homozygous for the transgene insertion. Both F1 and F2 animals are analyzed by Southern blotting to confirm the presence and copy number of the transgene, and blood samples analyzed for the presence of T, B and NK cell surface markers and NK function as described in Section F, above and in Example 3, Section F.

EXAMPLE 2

ζ-DT Hit and Run ES Cell Clones

One consequence of targeting the DT expression vector by an insertion vector is the introduction of a duplication of approximately 5.1 kb of CD3ζ sequence (Fig. 11) This duplication could lead to an intrachromasomal recombination that would loop out the DT gene and could lead to clonal escape of a cycling bone marrow stem cell. Depending upon the in vivo frequency of this reversion, clonal escape may or may not be a problem in hemizygous animals. The probability of reversion occurring prior to developmental expression of DT in cell ablation will be reduced - by its square - by breeding the transgene to homozygosity.

Intrachromasomal reversion could be avoided by using the replacement style homologous recombination vector (Fig. 9B).

More preferred is the use of a "Hit and Run" style vector that results in the incorporation of the DT gene without extraneous plasmid sequences or selectable

marker genes. This strategy is shown in Fig. 23. This procedure involves two isolation steps and takes advantage of the pr sence of both a positive (neo^r) and negative (TK) selectable marker. Both of these genes are positioned either 3' or 5' of first and second portions of DNA homology that are in turn separated by the expression sequence for DT. The first step involves an insertion style integration driven by linearizing the vector within the first or second portion of DNA homology. Targeted clones are screened using the same approximately 350 bp DNA probe as described in Example 1 (Fig. 23C). The integration event leads to a duplication of genomic sequences which flank either side of the DT gene. By allowing for intrachromosomal rearrangements in the absence of positive selection in G418, revertants are obtained in which the duplicated DNAs recombine on one or the other side of the DT gene. The revertants are isolated by selection in FIAU, which kills any non-recombining cells that still retain a functional TK gene. Twelve primary, or "Hit", events from electroporating ES cells have been characterized. From these, ten "Run" clones have been generated that are characterized by: (1) cloning from back-selection in FIAU; (2) the presence of an intact junction between the CD3ζ promoter and DT gene; and (3) absence of DNA for neo^r, TK gene, and plasmid sequences, all of which would be deleted due to an intrachromosomal recombination event between duplicated genomic sequences present downstream of the DT gene (see Fig. 23). Four of these "Run" clones were injected into blastocysts, which result in animals for testing for the lack of mature T, B, and NK cells as in Example 1.

EXAMPLE 3

Inactivation of the Murine CD3-ζ Chain Gene in ES Cells

A. Genomic Sequences

Genomic BamHI DNA fragments spanning exons 2-8 of murine CD3-ζ chain gene were isolated as described in Example 1 (Fig. 2). pPGKneo (as described in Example 1) was modified to remove the pGK polyadenylation sequence for use in polyadenylation minus targeting vectors and pMCITK was similar to that used by Zijlstra, et al. (1989) Nature 342:435-438.

B. Homologous Recombination Vector for the Targeted Disruption of the CD3-ζ Chain Gene: Poly-A Minus Vector

The strategy for the design of a replacement vector for the targeted disruption of the murine CD3-ζ chain gene is based upon that of M. Capecchi, et al. (Mansour, S.L. (1988) Nature 336:348-352). The 4.2 kb and 2.3 kb BamHI fragments of CD3ζ (fragments 4.2 and 2.3 in Fig. 2) were subcloned into the BamHI site of pUC18 (Fig. 12, step 1). The pUC18 plasmid containing the 2.3 kb BamHI fragment was partially digested with BamHI and made blunt-ended with the Klenow fragment of DNA polymerase I (Fig. 12, step 2). A synthetic DNA linker was prepared that contains an internal Pst I site, stop codons in all three reading frames, and single-stranded ends that are complementary to the 5'-extensions of EcoRI cut DNA 9 (Fig. 12, step 3). The 4.2 kb BamHI fragment was cut out of pUC18 using HindIII and EcoRI and the PGKneo (poly-A$^-$) expression cassette was cut out of pGEM7(KJ1)SalI.polyA$^-$ (Fig. 12, step 4). A plasmid with blunt-ended 5'-end BamHI site (Fig. 12, step 2) was used to excise the 2.3 kb fragment using SalI and SmaI, followed by ligation into the same sites of pMH5/4.2neoA$^-$ (Fig. 12, step 5). The resultant plasmid, designated p4.2ζA$^-$2.3, is used to

electroporate ES cells for deriving homologous recombination events that insert the neo-expression cassette into the CD3ζ genomic locus.

A second version of this vector was constructed in which a TK expression unit was placed at the end of the 4.2 kb BamHI fragment of p4.2ζA⁻2.3. First, the HindIII site located in IVS 2, approximately 0.8 kb downstream of the BamHI site in exon 2, was blunt-ended using Klenow in order to facilitate subsequent cloning procedures (Fig. 12, step 6). This plasmid is designated pζA-ΔH3. Next, MCITK was subcloned as a BamHI to HindIII fragment into the BglII and HindIII sites of pMH2 to generate pMH2MCITK (Fig. 12, step 7). The insert from pζA⁻ΔH3 (Fig. 12, step 6) was excised using HindIII and SfiI and subcloned into the same sites of pMH2MCITK to form pζA⁻ΔH3TK (Fig. 12, step 8). In the final step, pζA⁻ΔH3TK was partially digested with BamHI, blunt-ended with Klenow, and self-ligated to yield pζA⁻TK$_f$. This step eliminates the final BamHI site flanking the 2.3 kb CD3ζ fragment.

C. Isolation of ES Cell Clones Containing Homologous Recombination of the CD3-ζ Inactivation Vector

The electroporation and selection of G418^r or G418^r + FIAU^r clones is described in section C of Example I.

D. Characterization of Homolgous Recombination of PGKneo into the CD3ζ Gene Locus of Murine ES Cell Clones

The homologous recombination strategy for disrupting the endogenous CD3ζ gene is diagramed in Fig. 13. Plasmids p4.2ζA⁻2.3 and pζA-TK$_f$ are shown at the top. They are aligned over the genomic map of the CD3ζ gene. Marked are the approximate locations of BamHI (B) and XbaI (X) restriction sites as well as the size of their corresponding DNA fragments in kilobase pairs. Th

bottom map depicts the expected result of recombination between either of the two targeting vectors and CD3ζ. Note that the vector which lost only the BamHl site at the 5'-end of the 2.3 kb BamHI fragment (p4.2ζA⁻2.3), gives rise to a 3.6 kb BamHI fragment as a result of a random insertion event, while the pζA-TK_f vector yields a 3.6kb BamHI fragment from the result of a homologous recombination event within CD3ζ chain gene, or in rare cases, by integration immediately adjacent to a BamHI site located randomly within the genome.

Diagnostic Southern blotting for a homologous recombination event is performed by digesting approximately 10 μg of ES cell clone DNA with 100 units of either BamHI or XbaI overnight at 37°C. The enzyme reaction is then directly electrophoresed in 0.9% agarose gels in TBE buffer. The DNA is transferred to Genescreen nylon filters and hybridized as previously described.

Probes for hybridization consisted of either the 1.5 kb HindIII to BamHI fragment of the 2.3 kb BamHI fragment (designated the 1.5 kb probe), or the 0.3 kb BamHI to XbaI region of the 3.3 kb BamHI fragment (designated the 300 bp probe), shown in Fig. 13. Positive clones were identified by the presence of two separate hybridization patterns. The first consists of the appearance of a novel 3.6 kb BamHI fragment that hybridizes to the 1.5 kb probe (in addition to the endogenous 2.3 kb band). Clones derived from the transfection of p4.2ζA⁻2.3 contain such a band regardless of the site of integration as long as the PGKneoA+2.3 BamHI arm is intact. Clones derived from transfection of p4.2ζA⁻2.3TK obtain such a band as a result of homologous recombination or due to random integration adjacent to a BamHI site. The latter event is distinguished by the XbaI digest pattern. Only homologous recombinants yield a novel 2.6 kb XbaI

fragment capable f hybridizing to the 300 bp flanking probe. Confirmation of homologous recombination requires both diagnostic patterns be present in DNA from the same clone. Alternatively, the presence of a fusion RNA that hybridizes to both neoA⁻ and CD3ζ probe sequences downstream of exon 4 would indicate a targeted event.

ES clones containing an inactivated CD3ζ gene based upon this analysis are used to generate transgenic animals as described in Example 1.

E. Homologous Recombination Vector for the Targeted Disruption of the CD3ζ Chain Gene; Poly-A Plus Vector

Plasmid pζA-TK was further modified by linearizing the vector by partially digesting with BamHI (Fig. 12, step 8 for location of the two BamHI sites). The cohesive ends were made blunt-ended using Klenow, and the vector ligated to an approximately 300 bp fragment consisting of the polyadenylation sequence isolated from pSV40CAT. This vector was used to electroporate ES cells as in Section C, Example I, and the clones characterized by the Southern blot hybridization pattern outlines in Fig. 17. As probe, the 0.3 kb BamHI to XbaI DNA fragment (Section D of this Example) was hybridized to isolated ES cell DNA digested with XbaI. Targeted clones were identified at a frequency of approximately 1 in 7 doubly-selected clones (G418 and FIAU). Targeted clones contained a 3.3 kb endogenous band plus a novel 2.6 kb band. Chimeric mice were generated as described in Example 1 and most of these transmitted the targeted allele through the germ line. Heterozygous siblings were mated to derive homozygous offspring identified by the presence of two 2.6 kb targeted alleles (Fig. 17B).

F. Analysis of T, B and NK Cell Lineages in Transgenic Immunodeficient Mice

1. Lymphocyte Analysis

Homozygous CD3ζ knockout mice (-/-) were either bled or sacrificed at various ages for cellular analyses. Lymphocytes from the blood, spleen and thymus were stained for T, B, and NK cell surface markers.

Peripheral blood lymphocytes (PBLs) from four to five week old mice were analyzed for the presence of T, B, or NK cells. Cells were stained with monoclonal antibodies against mouse CD3ε (FITC-conjugate from Boehringer Mannheim), B220 (PE-conjugate from Pharmingen) and NK1.1 (PE-linked from Pharmingen).

Double staining patterns for B220 vs. CD3ε are shown in Fig. 18. In panel A, the +/+ wild type animal shows the normal single staining profile for populations of B and T cells. The ratio of B to T cells was approximately 1:1. In marked contrast, the -/- CD3ζ knock-out mouse (Fig. 18B) showed a complete absence of CD3ε positive cells and significantly fewer cells within the lymphocyte gate. This difference suggests that in the absence of CD3ζ, mature TCR-bearing cells fail to exit the thymus.

Staining with anti-NK1.1 antibody showed the presence of approximately 5-6% NK1.1 positive cells in two -/- mice (Figs. 18C and 18D). This suggests that the overall number of PBL-derived NK1.1 positive cells may not have been affected by the CD3ζ knock-out.

Cells from the spleens of 8-9 week old mice were then isolated from one +/+ and two -/- mice and stained for the presence of several T cell markers. Spleen cells stained for CD3ε vs. TCR α/β chains are shown in Fig.

19A. The +/+ mouse spleen contained approximately 22% double positive cells while none of the two mice contained cells displaying this TCR receptor pair and CD3ε. This result is consistent with staining of PBLs with B220 vs. CD3ε (Fig. 18) and indicates a failure of -/- mice to develop mature T cells.

Splenocytes stained with CD3ε vs. TCR γ/δ are shown in Fig. 19B. While all three mice show virtually no TCR γ/δ cells, 21% of the +/+ splenocytes are CD3ε positive. The -/- cells contain no CD3ε positive cells, consistent with the staining patten shown in Fig. 19A.

Wild type +/+ mice contain cells double positive for both TCR α/β and CD4 (6.2%) and TCR α/β and CD8 (9%) (Fig. 19C). The -/- mice contain no such double positive cells, but do have cells that stain exclusively for either CD4 (10-17%) or CD8 (4%). Normally, cells staining for either CD4 or CD8 also contain TCR α/β receptors. Additional staining could not detect CD4+ or CD8+ cells bearing TCR γ/δ receptors.

These results indicate that splenocytes from CD3ζ disrupted mice fail to develop normally into TCR-bearing lymphocytes. A small percentage of these cells display either CD4 or CD8, and there seem to be some cells that stain solely for TCR α/β. It is highly unlikely that these cells would possess the specificity of normal T cells due to a lack of MHC restriction. They may, however, represent a population of cells normally not seen or present at very low levels, but which provide a source of cytokines/cellular signals important to hematopoietic lineage development.

Staining of thymocytes from th same 8-9 week old mice is shown in Fig. 20. Thymi from -/- mice were approximately 0.2x normal size and produced 0.1x the number of cells as +/+ thymi. Normal thymocytes coexpressed TCRα/β and CD3ϵ (12%) while those from two -/-mice did not (Fig. 20A). The -/- thymocytes expressed relatively normal amounts of CD4 and CD8 (these are presumably double positive cells) yet they never develop into CD3ϵ positive cells (Figs. 20B,C). These results imply that the block to T cell differentiation in the -/- mice occurs at the CD4+CD8+ stage due to the inability to form functional TCR/CD3 complexes. The fact that a small number of CD4+ or CD8+ single positive cells appear in the periphery suggests that the CD4+CD8+ double positive cells in the thymus may stochastically down regulate one of the two proteins and escape the thymus. Alternatively, these cells may arise extrathymically.

2. Natural Killer Cell Activity

NK cell function is examined by standard ^{51}Cr-release assays (Biron, C.A. et al. (1987) J. Immunol. 139:1704-1710). Target cells are labeled with sodium chromate ^{51}Cr, NewEngland Nuclear). Three or more effector to target cell (E:T) ratios ranging from 0.3:1 to 11:1 are tested in microtiter plates with 10^4 targets/well. Assays are run from 4-6 hours at 37°C. Normal medium is added to target cells for spontaneous lysis determinations, and 1% Nonidet P-40 is added for determination of 100% lysis. Supernatant fluid is harvested to determine counts released from target cells. Target cells include the NK-sensitive line, YAC-1, as well as the adherent H-2k line, L-929, and the adherent H2b line, MC57G.

3. Immunohistology

Organs of the transgenic mice are examined and immunohistology carried out. The ER-TR series of antibodies (mouse thymic epithelium) are available for immunohistology.

EXAMPLE 4

Inactivtion of the Murine CD3-γ and δ Chain Genes in ES Cells

A. Genomic Sequences

Genomic clones spanning the entier CD3δ gene and 5'-end of CD3γ were provided by Dr. C. Terhorst, Dana Farber Cancer Institute (Saito, H. et al. (1987) Proc. Natl. Acad. Sci 84:9131-9134). The two genes are located adjacent to one another on mouse chromosome 9. They share a common intergenic region of approximately 1300 bp from which divergent transcription of the two genes is initiated. Clone pTm δ contains approximately 9 kb of murine genomic DNA starting from an EcoRI site 3' of the fifth and last exon of CD3δ and ending at a Pst I site approximately 3.4 kb into the first intervening sequence of CD3 γ. The restriction map for this clone is shown in Fig. 14.

B. Homologous Recombination Vector for the Targeted Disruption of the CD3γ and δ Chain Genes

Simultaneous inactivation of the two genes in ES cells is acheived by removing the genomic sequence lying between and including exons one of the two genes. Targeting vectors of this type are assembled as shown in Fig. 14. In the first version, plasmid pTmδ was digested with SpeI and filled in with Klenow. It was then cut to completion with BamHI and electrophoresed in a 1.0% agarose gel in TBE. A band of approximately 2.5 kb was excised and extracted with Gene Clean II.

A contiguous fragment II (EcoRI to BamHI) of approximately 2.5 kb (Fragment I in Fig. 14) was purified in a similar manner. The recipient vector, pMH4 has a modification of pMH2 (Example 1) in which the order of restriction sites in the polylinker was converted to that shown in Fig. 14. It was digested with Asp718 and treated with Klenow to blunt-end the Asp718 site, followed by digestion with EcoRI. A three part ligation was performed between the two pTmδ fragments and pMH4 as shown in Fig. 14, step 1, to form pMH4/5.0δ which contains approximately 5.0kb of CD3δ DNA. This sequence was used as one of the arms flanking the PGK-neo^r-expression cassette.

A 1.4 kb fragment from the CD3γ region was isolated by digesting pTmδ with Asp718 and EcoRV followed by gel purification. The PGK-TK expression cassette was removed from pGEM7(TK)SalI by digestion with EcoRI and treatment with Klenow, followed by SalI, and isolated by gel purification. This 2.7 kb fragment contains the PGK promoter operably linked to the herpes viral thymidine kinase gene and PGK polyadenylation sequence was ligated with the 1.4 kb CD3γ fragment into pUC18 (digested with Asp718 and SalI) to form pUC18/γ/TK (Fig. 14, step 2).

The PGKneo^r expression cassette was isolated from pGEM7(KJ1)SalI (containing a polyadenylation signal, Example 1) or pGEM7(KJ1)SalIpolyA$^-$ (lacking a polyadenylation signal, Example 3) by digestion with EcoRI and SalI. The 1.4 kb CD3γ fragment linked to PGKTK was isolated by digesting pUC18/γ/TK with EcoRI and SalI. A four part ligation among: (i) PGKneo^r, (ii) a synthetic oligonucleotide linker with compatible EcoRI arms, (iii) γ/TK, and (iv) pUC18 digested with SalI, was performed as in Fig. 14, step 3 to form pUC18/neoA+(or A-)/γ/TK. The arms of the synthetic

linker, while compatible with EcoRI digested ends, will not recut with EcoRI after ligation.

The neo/γ/TK fragment was then transferred from pUC18/neoA+ (or A-)/γ/TK to pKUN2 (a modified version of pKUN9 (Peeters, et al. (1986) Gene 41:39-46) in which the polylinker was modified to contain NotI and SfiI sites as shown in Fig. 14, step 4) by isolating the EcoRI to HindIII insert and ligating it into the respective sites of pKUN2 to form pKUN/neo/γ/TK (Fig. 14, step 4). This plasmid was used for the final ligation reactions with fragments of the CD3δ gene. Plasmid pMH4/5.0δ was cut with NotI and EcoRV to isolate the 5.0 kb CD3δ fragment. Plasmid pKUN/neoA+(or A-) was digested with EcoRI and treated with Klenow, and then digested with NotI. The two fragments were ligated together to form pMH4/5.0δ (Fig. 14, steps 5A and 5C). The final form of this vector thus contains the PGK-neo^r expression cassette (either with or without an adjacent polyadenylation sequence) flanked by 5.0 kb of CD3δ sequence on one side, and 1.4 kb of CD3γ sequence on the other side. The CD3γ sequence is in turn flanked by the PGKTK expression cassette. With respect to the presence or absence of a PGKneo polyadenylation sequence, these vectors are designated p5.0/neo A+(or A-)/1.4TK. Note that p5.0/neoA-/1.4/TK provides a polyadenylation site for the neo^r transcript in the form of a fusion RNA molecule between neor and CD3δ; the polyadenylation site is located at the end of exon 5.

Poly-A Minus Vector

In order to construct a vector in which there is no polyadenylation site provided for the neo^r transcript by the vector sequence, the CD3δ fragment was truncated prior to ligation into pKUN/neo/γ/TK (Fig. 14, step 5B). This was then accomplished by digesting

pMH4/5.0δ with BamHI (located just upstream of CD3δ exon 2) and treating with Klenow, followed by digesting with MluI. The approximately 2.5 kb fragment was isolated and subcloned into pMH4 cut with EcoRI (and blunt-ended with Klenow) and MluI to form pMH4/2.5δ. The 2.5 kb CD3δ fragment was isolated as a NotI to EcoRV fragment and then ligated into the NotI and blunt-ended EcoRI sites of pKUN/neoA-/γ/TK to form pKUN/2.5/neoA-/1.4TK (Fig. 14, step 5C).

C. Isolation of ES Cell Clones Containing Homologous Recombination of the CD3-γ/δ Inactivation Vector

The electroporation and selection of G418^r clones is described in section C of Example I. For double selection of ES cells, FIAU (Oclassen, San Rafael, California) is added at a concentration of 0.5 x 10^{-6}M.

D. Characterization of Homolgous Recombination of PGKneo into the CD3γ/δ Gene Locus of Murine ES Cell Clones

Genomic DNA from G418^r/FIAU^r ES cell clones was prepared as described in Example I. Approximately 10 μg of total DNA is digested with 100 units of Pst I overnight at 37°C in 20 μl buffer supplied by the manufacturer (either BM or NEB). The reaction mixture is electrophoresed directly into 0.9% agarose gels in TBE. DNA transfer to nylon filters and hybridization conditions is the same as described in Example I.

Probe DNA consists of approximately 450 bp of genomic DNA between the 3'-end Pst I site (with respect to CD3γ) in pTmδ and the first upstream Pvu II site (Fig. 15). Digestion of genomic DNA at the endogenous Pst I sites gives rise to an approximately 8.0 kb fragment, while a homologous recombinant gives rise to an approximately 2.0 kb band due to the introduction of a novel Pst I site at the junction of the CD3γ fragment

and the PGK-neo^r sequence (Fig. 15). ES cell clones showing this pattern are subsequently analyzed using restriction enzyme digests of either Pst I or Pvu II alone, or together, and probed with the 1.4 kb CD3γ arm fragment. The endogenous and homologous recombinant DNAs give the following banding patterns after hybridization to the 1.4 kb probe:

Band sizes expected (kb) from enzyme(s) listed			
DNA	Pst I alone	Pvu II alone	Pst I + Pvu II
Endogenous	8.0	1.9	1.9
Recombinant (replace-ment)	2.0	2.1	1.6

ES cell clones with genotypes displaying the above Southern blotting patterns are expanded and used for injection into recipient blastocysts to form germline chimeras as described in Example I.

E. Generation of Germline Chimeras Containing Gene Inactivation of the CD3γ/δ Chain Genes

Male founders from the ES cell blastocyst injections in Section D, above, are bred with C57/BL6 females to generate offspring that are 100% agouti and tested for germline transmission of the introduced transgene. DNA isolated from tail samples is prepared by digestion in proteinase K and analyzed by Southern blot hybridization as in Section D to confirm the presence of the transgene. Siblings from this generation are in turn bred to produce mice that are homozygous for the inactivated CDγ/δ genes.

F. Preliminary Analysis of T, B. and NK Cell Lineages in Transgenic Immunodeficient Mice

See Example 1, Section F, and Example 3, Section F.

The foregoing description of the preferred embodiments of the present invention has been presented for purposes of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise form disclosed, and many modifications are variations are possible in light of the above teaching. Such modifications and variations which may be apparent to a person skilled in the art are intended to be within the scope of this invention.

WHAT IS CLAIMED IS:

1. A transgenic non-human animal having a genotype comprising a DNA sequence encoding a dominant lethal polypeptide operably linked to an expression regulation sequence of a lymphoid gene.

2. The transgenic non-human animal of Claim 1 wherein said expression regulation sequence is of a lymphoid gene expressed by all T lymphocytes.

3. The transgenic non-human animal of Claim 1 wherein said expression regulation sequence is selected from the group consisting of expression regulation sequences of CD1, CD2, CD3-γ,CD3 CD3-δ, CD3-ϵ, CD3-ζ, CD3-η, CD5, CD7, $p56^{lck}$, RAG-1, RAG-2, IL-2Rβ chain, J11d and fyn genes.

4. The transgenic hon-human animal of Claim 3 wherein said expression regulation sequence is of a gene encoding CD3ζ.

5. The transgenic non-human animal of Claim 2 having a phenotype characterized by a substantial immunodeficiency of at least one function of a T lymphocyte otherwise present in the species from which said transgenic non-human animal is derived.

6. The transgenic non-human animal of Claim 1 wherein said expression regulation sequence is of a lymphoid gene expressed by a B lymphocyte.

7. The transgenic non-human animal of Claim 6 wherein said first expression regulation sequence is selected from the group consisting of expression regulation sequences of RAG-1, RAG-2, Ig-β, IgM-α, J11d, CD19, CD20, CD38, CD40, CD45, CD72, CD76 genes and genes

associated with the immunoglobulin isotype Igμ, Igδ. Igγ, Igα, Igϵ, Igκ and Igλ.

8. The transgenic non-human animal of Claim 6 having a phenotype characterized by the substantial immunodeficiency of at least one function of a B lymphocyte otherwise present in the species from which said transgenic non-human animal is derived.

9. A transgenic non-human animal having a genotype comprising a first DNA sequence encoding an expression regulation sequence of a lymphoid gene expressed by NK cells operably linked to a second DNA sequence encoding a lethal polypeptide.

10. The transgenic non-human animal of Claim 5 wherein said first DNA sequence is selected from the group consisting of expression regulation sequences of CD2, CD3-ζ, p56^{lck}, fyn, NK1, NK2, CD56, Fc$_\epsilon$RI-γ and IL-2Rβ genes.

11. The transgenic non-human animal of Claim 10 wherein said expression regulation sequence is of a CD3ζ gene.

12. A transgenic non-human animal of Claim 9 having a phenotype characterized by a substantial immunodeficiency of at least one function of an NK cell otherwise present in the species from which said transgenic non-human animal is derived.

13. A transgenic non-human animal having a genotype comprising a first DNA sequence encoding an expression regulation sequence for a lymphoid gene expressed by a large granular lymphocyte operably linked to a second DNA sequence encoding a lethal polypeptide.

14. The transgenic non-human animal of Claim 13 wherein said first DNA sequence is selected from the group consisting of expression regulation sequences of αTCAR, βTCAR, NK1, FcγRIII and FcεRI-γ genes.

15. The transgenic non-human animal of Claim 13 having a phenotype characterized by the substantial immunodeficiency of at least one function of a large granular lymphocyte otherwise present in the species from which said transgenic non-human animal is derived.

16. A transgenic non-human animal having a genotype characterized by a transgene comprising a first DNA sequence operably linked to a second DNA sequence encoding a lethal polypeptide, wherein said first DNA sequence is selected from the group of expression regulation sequences of CD1, CD2, CD3-γ, CD3-δ, CD3-ε, CD3-ζ, CD3-η, CD4, CD5, CD7, CD8,, CD19, CD20, CD38, CD40, CD45, CD72, CD76, $p56^{lck}$, IL-2Rβ, J11d, fyn, NK1, NK2, FcγRI-γ, IL-2Rβ, αTCAB, βTCAR, γTCAR, δTCAR, FcγRIII, RAG-1, RAG-2, Ig-β, IgM-α genes and genes associated with the immunoglobulin isotypes Igμ, Igδ, Igγ, Igα, Igε, Igκ and Igλ.

17. The transgenic non-human animal of Claim 1, 9, 13, or 16 wherein said second DNA sequence is integrated into an endogenous allele of a gene encoding said expression regulation sequence such that the expression of said second DNA sequence is controlled by said expression regulation sequence.

18. The transgenic non-human animal of Claim 1, 9, 13 or 16 wherein a transgene encoding said expression regulation sequence and said lethal polypeptide is randomly integrated into the genome of said non-human animal.

19. A transgenic non-human animal having a genotype charact rized by the substitution, deletion or insertion of one or more nucleotides in an endogenous allele of at least one CD3-type gene.

20. The transgenic non-human animal of Claim 19 wherein said CD3-type gene encodes a CD3-γ, CD3-δ, CD3-ϵ, CD3-η or CD3-η polypeptide.

21. The transgenic non-human animal of Claim 19 wherein said CD3-type gene encodes a CD3ζ polypeptide.

22. The transgenic non-human animal of Claim 19 wherein said substitution, deletion or insertion in said CD3-type gene causes disruption in the production of a functional gene product encoded by said endogenous allele.

23. The transgenic non-human animal of Claim 19 wherein said genotype confers a phenotype characterized by a substantial immunodeficiency in at least one function of a T lymphocyte.

24. A transgenic immunodeficient animal having at least a first genotype conferring a first immunodeficient phenotype and a second genotype conferring a second immunodeficient phenotype, wherein at least one of said genotype is formed upon genomic integration of a transgene encoding said at least one genotype.

25. A transgenic immunodeficient non-human animal containing a xenograph and characterized by a phenotype wherein said animal has an enhanced ability to maintain said xenograph as compared to the maintenance of the xenograph in the species of animal from which said transgenic animal is derived.

26. An animal cell having a genotype comprising a first DNA sequence encoding an expression regulation sequence operably linked to a second DNA sequence encoding a lethal polypeptide, wherein said first DNA sequence is selected from the group of expression regulation sequences of CD1, CD2, CD3-γ, CD3-δ, CD3-ε, CD3-ζ, CD3-η, CD4, CD5, CD7, CD8,, CD19, CD20, CD38, CD40, CD45, CD72, CD76, $p56^{lck}$, IL-2Rβ, J11d, fyn, NK1, NK2, FcγRI-γ, IL-2Rβ, αTCAB, βTCAR, γTCAR, δTCAR, FcγRIII, RAG-1, RAG-2, Ig-β, IgM-α genes and genes associated with the immunoglobulin isotypes Igμ, Igδ, Igγ, Igα, Igε, Igκ and Igλ.

27. The animal cell of Claim 26 wherein said expression regulation sequence is of a gene encoding CD3ζ.

28. An animal cell having a genotype characterized by the substitution, deletion or insertion of one or more nucleotides in an endogenous allele of at least one CD3-type gene.

29. The animal cell of Claim 28 wherein said CD3-type gene encodes a CD3-γ, CD3-δ, CD3-ε, CD3-η or CD3-ζ polypeptide.

30. The animal cell of Claim 29 wherein said CD3-type gene encodes a CD3ζ polypeptide.

31. A recombinant nucleic acid comprising a first sequence encoding an expression regulation sequence operably linked to a second sequence encoding a lethal polypeptide, wherein said first sequence is selected from the group of expression regulation sequences of CD1, CD2, CD3-γ, CD3-δ, CD3-ε, CD3-ζ, CD3-η, CD4, CD5, CD7, CD8,, CD19, CD20, CD38, CD40, CD45, CD72, CD76, $p56^{lck}$, IL-2Rβ, J11d, fyn, NK1, NK2, FcγRI-γ, IL-2Rβ,

αTCAB, βTCAR, γTCAR, δTCAR, FcγRIII, RAG-1, RAG-2, Ig-β, IgM-α genes and genes associated with the immunoglobulin isotypes Igμ, Igδ, Igγ, Igα, Igε, Igκ and Igλ.

32. The nucleic acid of Claim 31 wherein said expression regulation sequence is of a gene encoding CD3ζ.

33. The nucleic acid of Claim 31 randomly integrated into the genome of a non-human animal.

34. The nucleic acid of Claim 31 integrated into a predetermined endogenous allele of a gene encoding said expression regulation sequence.

35. A recombinant nucleic acid comprising a first sequence having first and second portions and a second sequence positioned between said first and second portions encoding a toxic polypeptide, wherein said first and second portions are capable of homologous recombination with a predetermined endogenous allele in the genome of a non-human animal such that expression of said second sequence is under control of an expression regulation sequence selected from the group consisting of expression regulation sequences of CD1, CD2, CD3-γ, CD3-δ, CD3-ε, CD3-ζ, CD3-η, CD4, CD5, CD7, CD8,, CD19, CD20, CD38, CD40, CD45, CD72, CD76, $p56^{lck}$, IL-2Rβ, J11d, fyn, NK1, NK2, FcγRI-γ, IL-2Rβ, αTCAB, βTCAR, γTCAR, δTCAR, FcγRIII, RAG-1, RAG-2, Ig-β, IgM-α genes and genes associated with the immunoglobulin isotypes Igμ, Igδ, Igγ, Igα, Igε, Igκ and Igλ.

36. A recombinant nucleic acid comprising a first sequence encoding the substitution, deletion or insertion of one or more nucleotides in at least a part of a CD3-type gene, said first sequence comprising

first and second sequence portions capable of homologously recombining with an endogenous CD3 allele.

37. The nucleic acid of Claim 36 wherein said CD3-type gene is CD3ζ.

38. A method of producing a transgenic non-human animal having a first phenotype characterized by a substantial immunodeficiency comprising

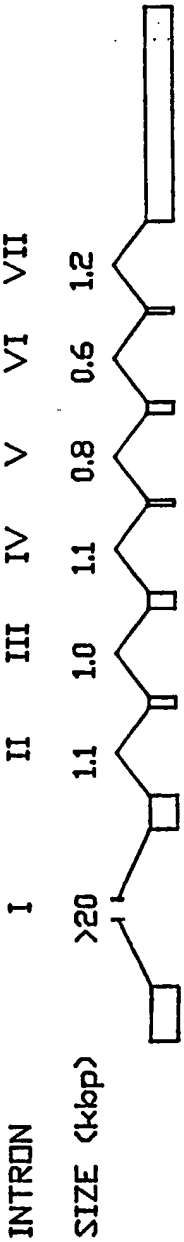
introducing a recombinant nucleic acid of any of Claims 31-37 into an embryonal target cell of a non-human animal;

transplanting the transformed embryonal target cell formed thereby into a recipient parent, and

identifying offspring incorporating said nucleic acid into its genome.

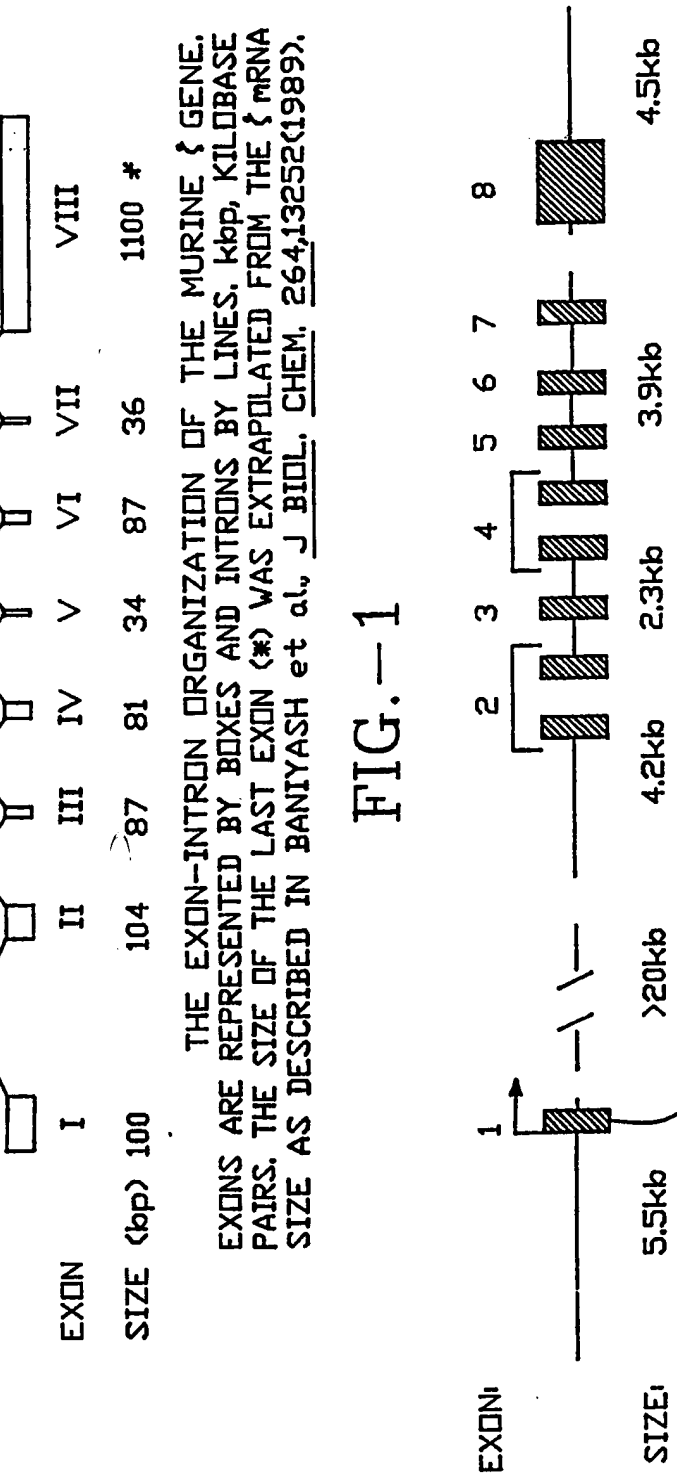
39. The method of Claim 38 wherein said recombinant nucleic acid is capable of homologous recombination with the genome of said embryonal target cell and said method further comprises prior to said transplanting the step of selecting an embryonal target cell into which said nucleic acid has integrated by homologous recombination.

40. A method of using a transgenic non-human animal having a substantial immunodeficiency in at least one immune function comprising introducing a xenograph into said transgenic non-human animal.



THE EXON-INTRON ORGANIZATION OF THE MURINE ζ GENE. EXONS ARE REPRESENTED BY BOXES AND INTRONS BY LINES. kbp, KILOBASE PAIRS. THE SIZE OF THE LAST EXON (*) WAS EXTRAPOLATED FROM THE ζ mRNA SIZE AS DESCRIBED IN BANIYASH et al., J BIOL. CHEM. 264,13252(1989).

FIG.—1



BamHI RESTRICTION MAP OF THE MURINE CD3 ζ GENE. EXONS 2 AND 4 HAVE INTERNAL BamHI SITES. ARROW INDICATES START OF TRANSCRIPTION.

FIG.—2

CCCACAGTCCTCCACTTCCTGGGGTGTCAGCCACAGAACAAAGCCAGCAGAGACTCCATCAGCGCCTCCT

TTTCTCCTCATCCTCCCAGGCATAGCTGCCTCTCTGCCTCTGGGTACCATCCCAGGGAAGCAGAAG

ATGAAGTGGAAAGTGTCTGTTCTCGCCTGCATCCTCCACGTGCGGTTCCCAGGAGCAGAGGCACAGAGCT

TTGGTCTGCTGGATCCCAAACTCTGCTACTTGCTAGATGGAATCCTCTTCATCTACGGAGTCATCATCAC

AGCCCTGTACCTGAGAGCAAAATTCAGCAGGAGTGCAGAGACTGCTGCCAACCTGCAGGACCCCAACCAG

CTCTACAATGAGCTCAATCTAGGGCGAAGAGAGGAATATGACGTCTTGGAGAAGAAGCGGGCTCGGGATC

CAGAGATGGGAGGCAAACAGCAGAGGAGGAACCCCCAGGAGGGCGTATACAATGCACTGCAGAAAGA

CAAGATGGCAGAAGCCTACAGTGAGATCGGCACAAAAGGCGAGAGGCGGAGAGGGGCACGATGGC

CTTTACCAGGGTCTCAGCACTGCCACCAAGGACACCTATGACGCCTTGCATATGCAGACCCTGGCCCCTC

GCTAACAGCCAGGGCATTTCTCCCTCACGCGCTTCACCTGCTGATGTCACTTGTGAAGAACAGAGGACAA

AGCCCCCCTCAGTTTATTCATTTCCCAGCCACCATTTCATGACGAGGATGGTTCTCTCACTTGCCACATT

TGTCTTCTTCAGTTCCAGAGCACTGAACACAGAACGTCATCCCTGGACTCTCTAAAGGGAGAGCCACCCT

TGCTCTTCCACCCCAGCCCTGCTCTTGGGTCTTCTGGCAGGCTCCTCTCCTTGCAGAGCCCAGCCCTAGC

TAGGAGTTGGGGGGTGGAGGGTGGGGCACTAACACACTCCCTCCTGCAGCTAGCTGAGTTCACTTTGCTTT

GTAAAGTCCCCAGAGAAGCCCTAGGTACTGTGTGTATTGTTCTATGGGTATTGACTCGCTCCGCTCCTGC

FIG.—3A

TGTAAATTTGGCTTCTGTTGTCACACTTTGCAGTGTTGAGGTA-CATGTAATTAGGCCACATTGTGAAAG

GCAGAGAGGCAGGTACAAGGGAGTCCAGGTAAATGACAGCCAGAGGTGGCTCAAAGAAGGGAAGCAACAC

ACAAGGAAGGTTCCTAGCCACAGGGGAACAGTAACAAGGGGCCTCTTTCCATCAAGATCCTTTCCACAT

CTCCCAGCCTTTGTGCCAGGCACTCCAGGAACGGTAAGATGTCTTAGGTTCATGAAGACCATGATTTTAA

GAGTGCAAATAACTTGTGTACAAAATGACACAGAAAATAGGATGGGCCATTACCGCCAACGACAAGGGAG

CAAGAGAAAAGGGAGCAAGTCCTGCCTTCTTCCAGGAACTCGGTTCATCCCTGTCAGGTGGCAGGAAGAC

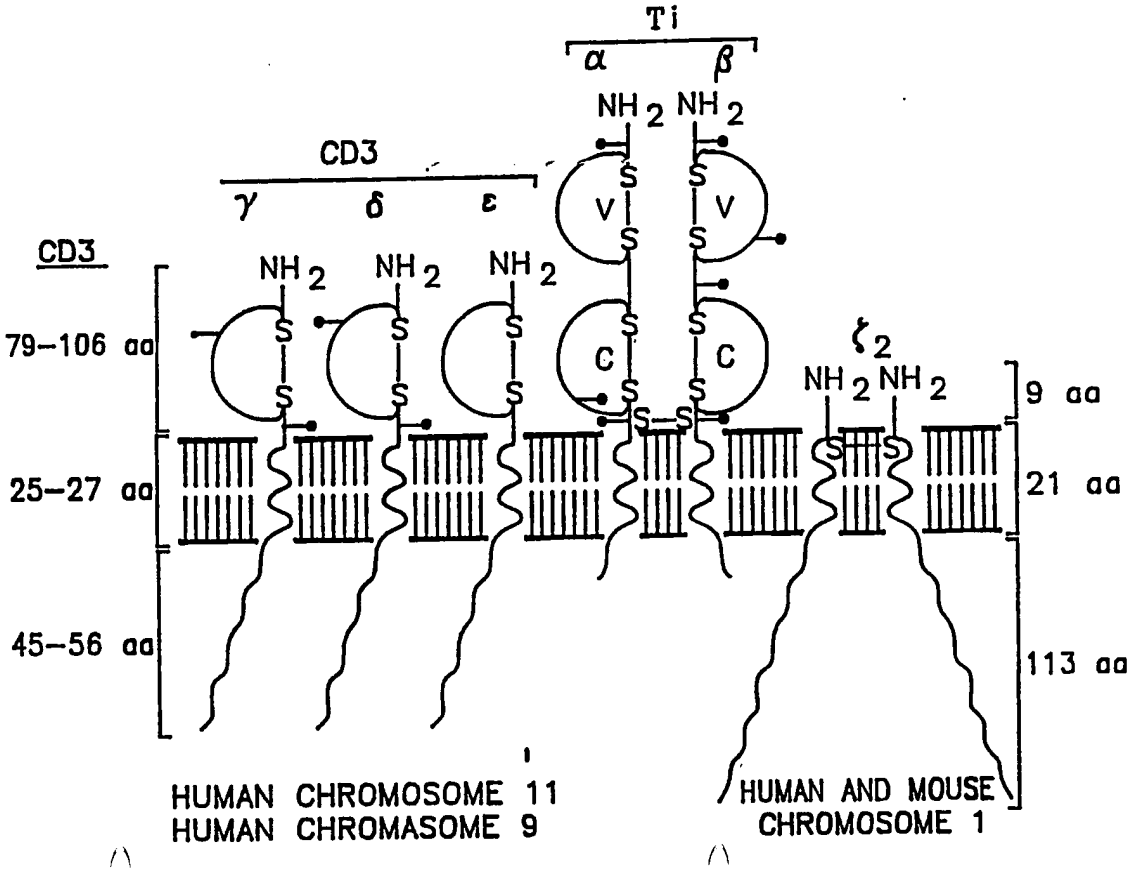
CCAGACCACCCAGGTCTGCAACGTTCCCAGTGACAATCAGTGGCCGGCAAGGTCATTGTGTCATTAAAGT

GCCTGTACCCATGGATGGGGAACCTCTGATCGCCTGGGTTTTTTAATTTATACCAGCTTGCTATTTTGCA

TAATAAACACTTTGCTAAAAATGATCTTTGAAGCCTTATTTCAGTGGTGAAGCCTGGGGTAGCAATG

FULL-LENGTH cDNA SEQUENCE OF THE MURINE TCR ζ CHAIN. THE TRANSCPIPTION INITIATION SITES ARE DENOTED BY DOTS, THE TRANSLATIONAL START AND STOP SITES AND THE POLYADENYLATION SIGNAL BY BOXES, AND INTRONS BY ARROWS. THE DIFFERENT REGIONS OF THE ζ PROTEIN ARE INDICATED: SIGNAL PEPTIDE (———), EXTRACELLULAR DOMAIN (~~~~~), TRANSMEMBRANE DOMAIN (———), AND THE CYTOPLASMIC TAIL (~~~~~). FROM BANIYASH et al., J BIOL. CHEM. 264,13252(1989).

FIG.-3B



STRUCTURE OF THE T CELL ANTIGEN RECEPTOR. THE SEVEN CHAINS FOUND IN THE MAJORITY OF T CELL RECEPTOR COMPLEXES ARE SHOWN IN SCHEMATIC FORM. THE DISTRIBUTION OF MASS OF EACH SUBUNIT WITH RESPECT TO T E MEMBRANE IS DRAWN ACCORDING TO THE PREDICTIONS BASED ON cDNA SEQUENCES. THE EXTERNAL DOMAINS OF THE TI AND CD3 COMPONENTS ARE DRAWN TO DEMONSTRATE THEIR IMMUNOGLOBULIN–LIKE DOMAIN STRUCTURES. AC, AMINO ACID. FROM BANIYASH et al., J. BIOL. CHEM. 264,13252(1989).

FIG.—4

CD3 ZETA CHAIN TARGETING APPROACH: SEQUENCE INSERTION VIA REPLACEMENT-TYPE VECTOR

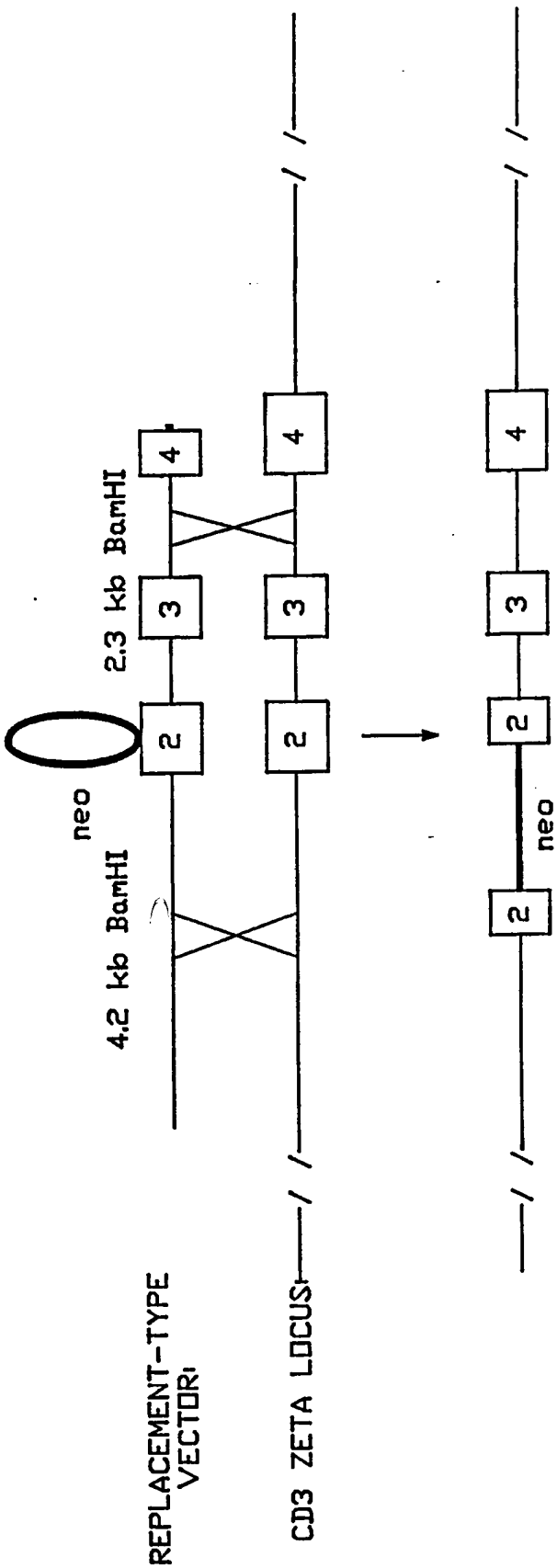


FIG.-5A

CD3 ZETA CHAIN TARGETING APPROACH: SEQUENCE DELETION VIA REPLACEMENT-TYPE VECTOR

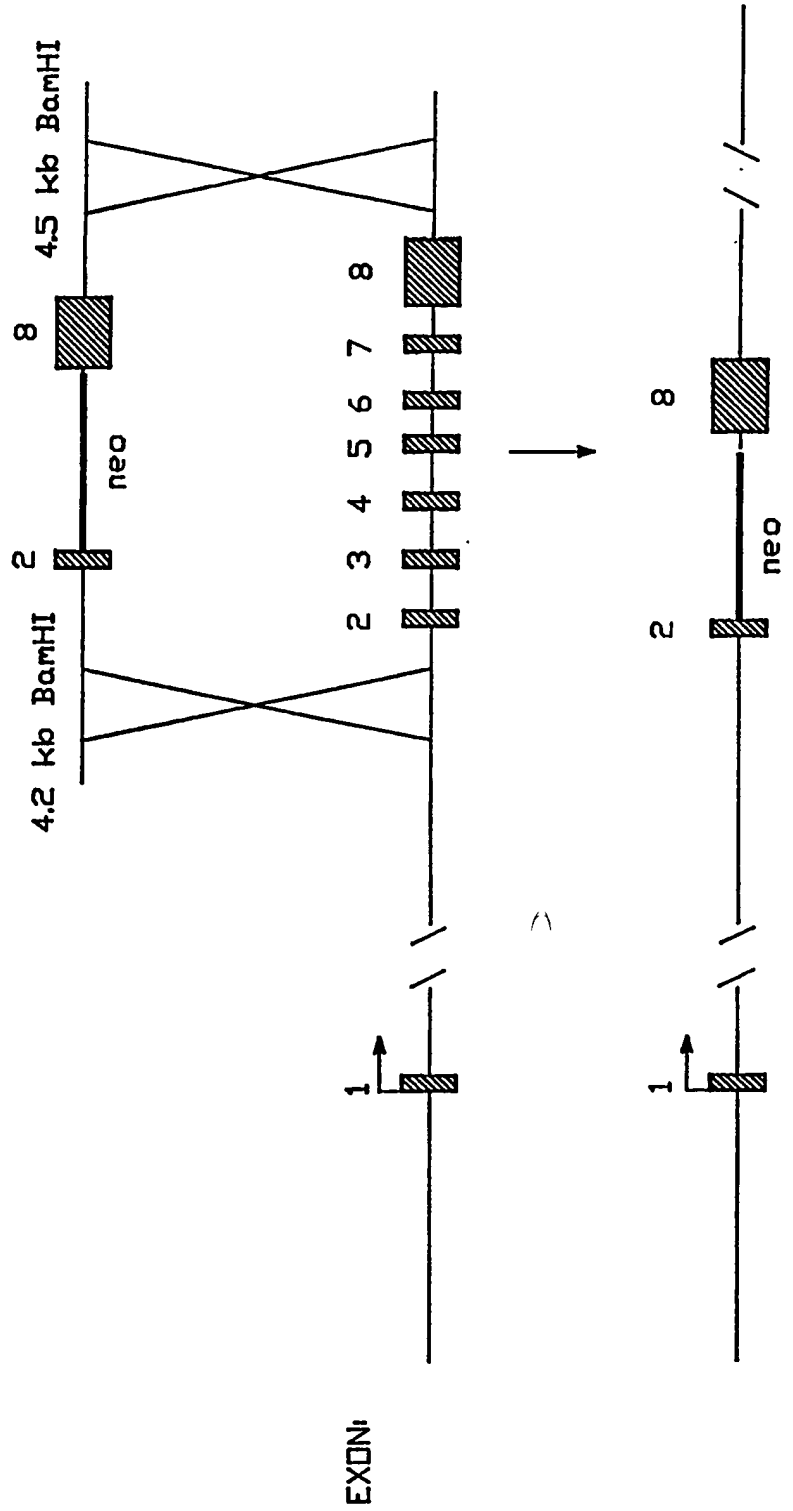


FIG.—5B

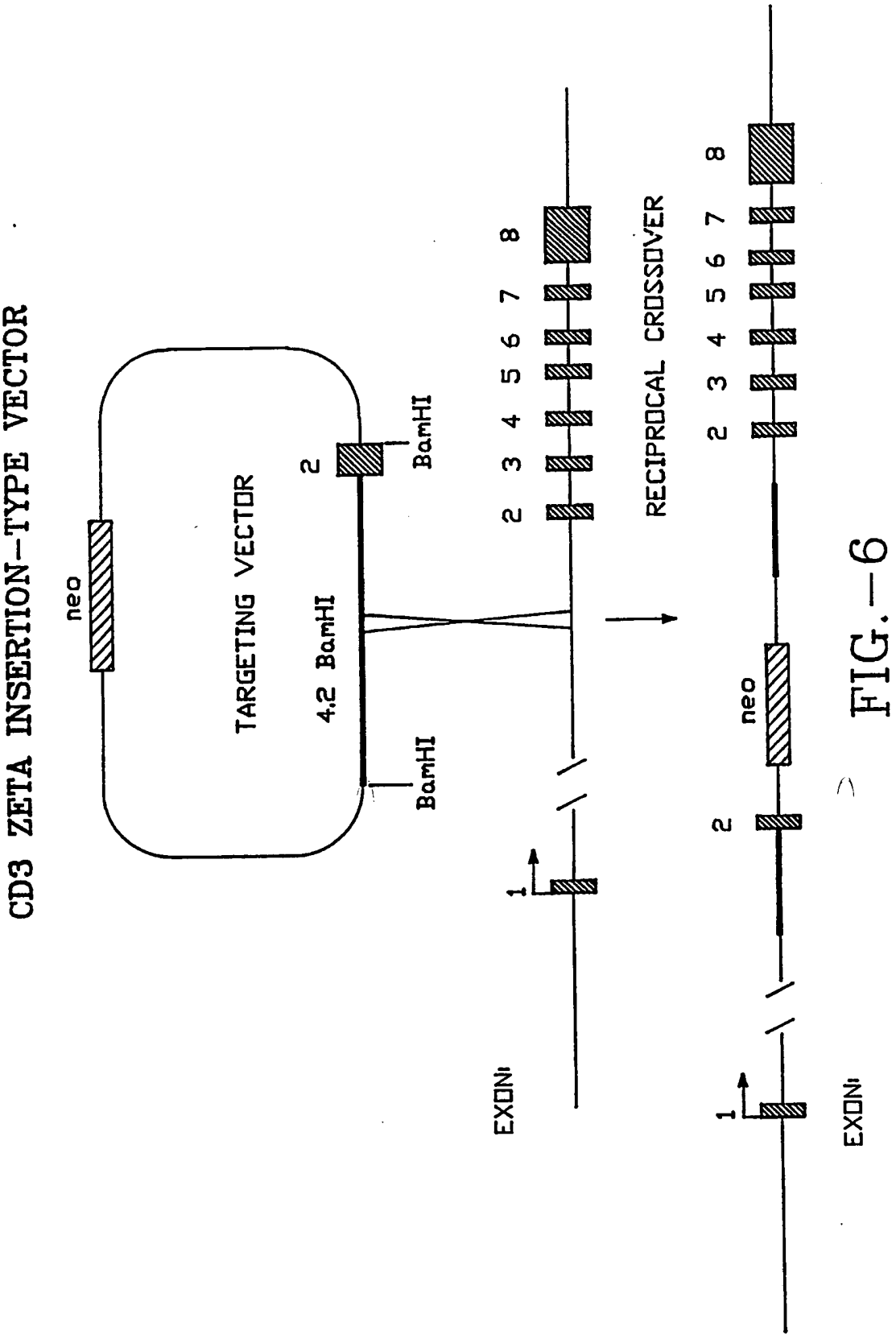

CD3 ZETA INSERTION-TYPE VECTOR
neo
TARGETING VECTOR
2
4.2 BamHI
BamHI
BamHI
2 3 4 5 6 7 8
1
EXON1
RECIPROCAL CROSSOVER
2 3 4 5 6 7 8
neo
2
1
EXON1

FIG.-6

CD3 ZETA CHAIN TARGETING APPROACH: SEQUENCE INSERTION VIA REPLACEMENT-TYPE VECTOR

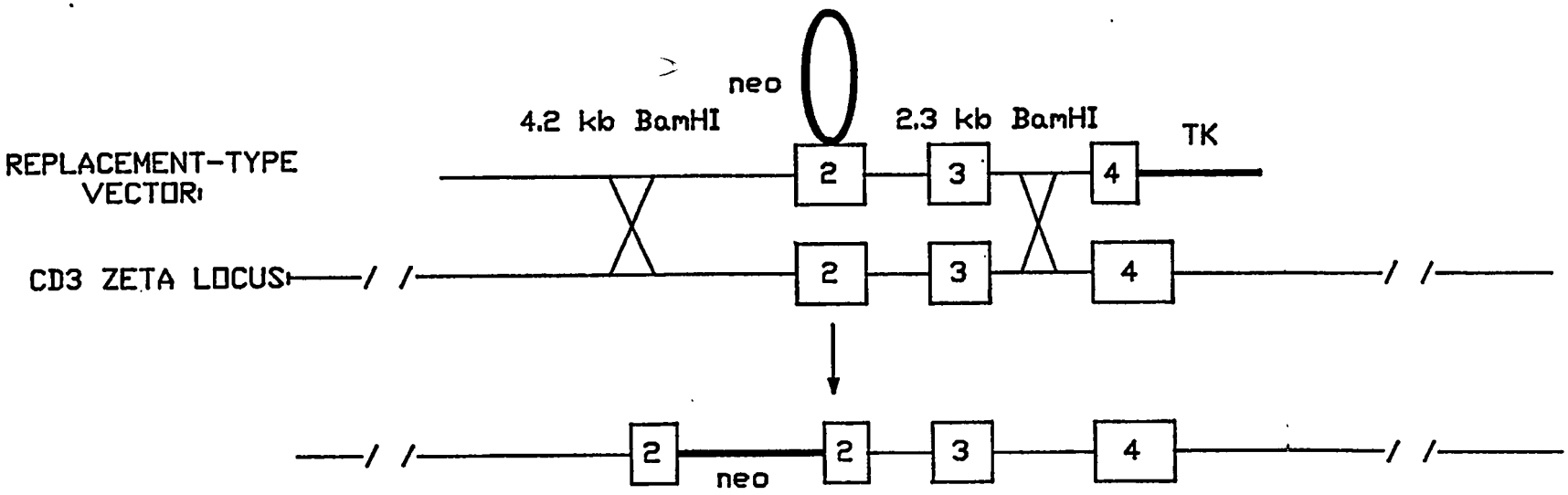


FIG.-7

CD3 ZETA DT-A/hGH MINIGENE

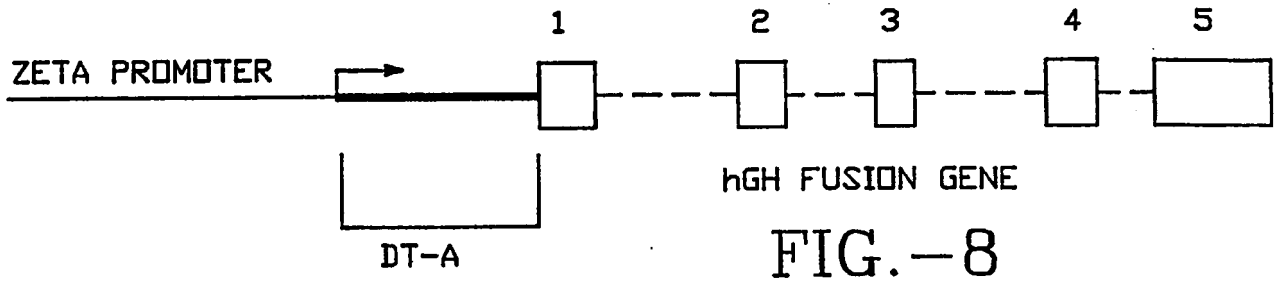


FIG.-8

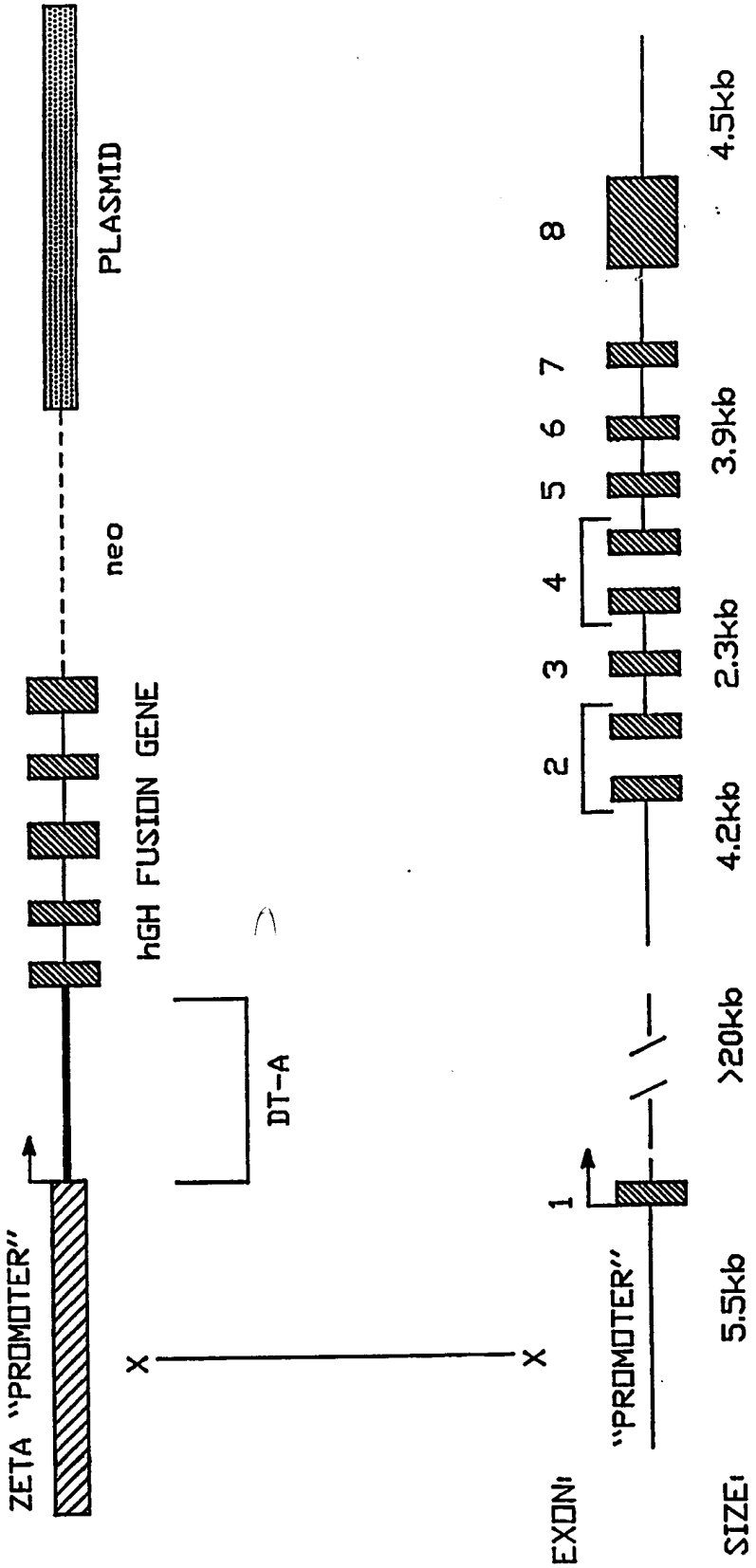

CD3 ZETA DT-A/hGH MINIGENE
PLASMID
neo
hGH FUSION GENE
DT-A
ZETA "PROMOTER"
X
X
EXON:
SIZE:
1
"PROMOTER"
5.5kb
>20kb
2
3
4
5
6
7
8
4.2kb
2.3kb
3.9kb
4.5kb
CD3 ZETA GENE

FIG.—9A

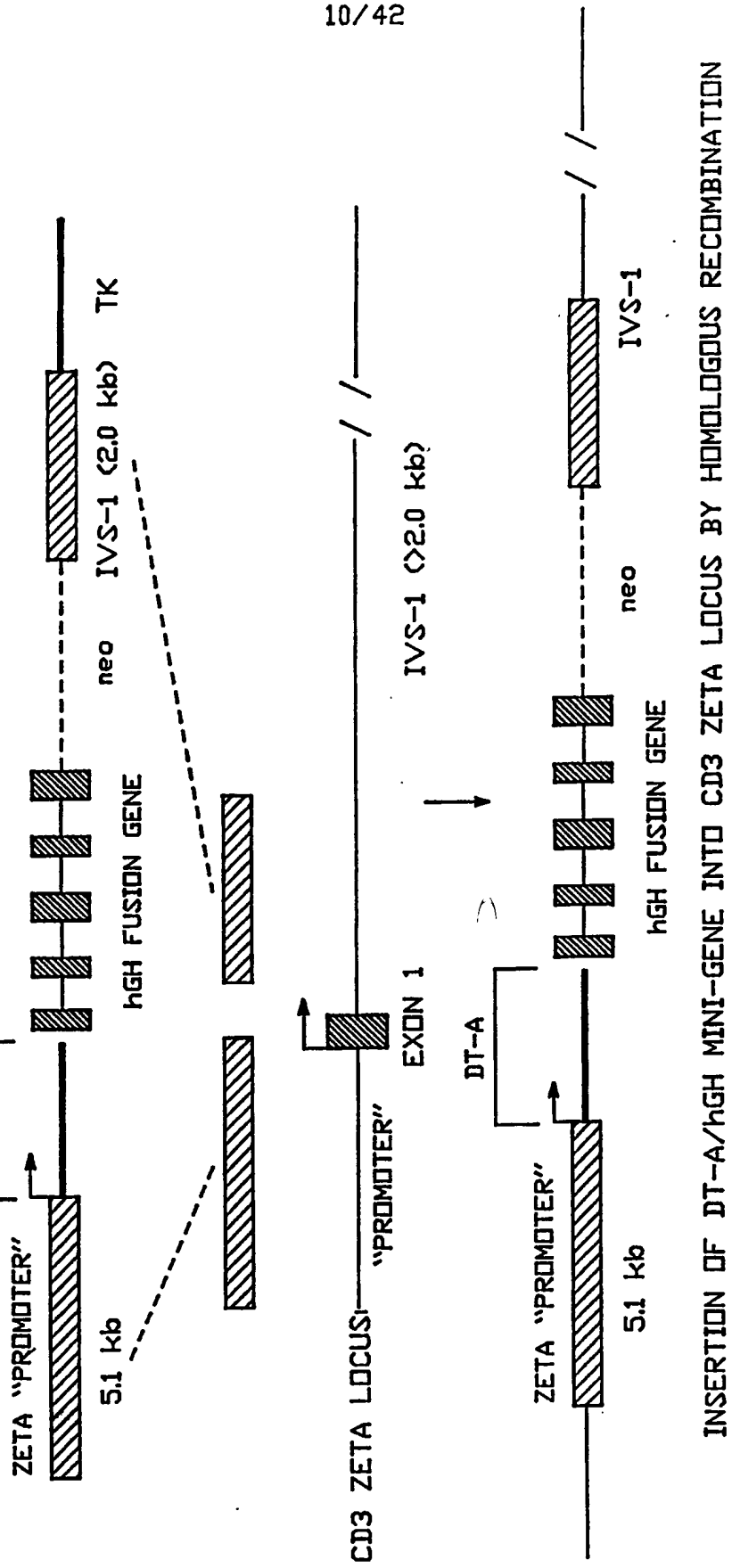

CD3 ZETA DT-A/hGH MINIGENE: REPLACEMENT-TYPE VECTOR
DT-A
ZETA "PROMOTER"
5.1 kb
neo
IVS-1 (2.0 kb)
TK
hGH FUSION GENE
CD3 ZETA LOCUS
"PROMOTER"
EXON 1
IVS-1 (>2.0 kb)
DT-A
ZETA "PROMOTER"
5.1 kb
neo
IVS-1
hGH FUSION GENE
INSERTION OF DT-A/hGH MINI-GENE INTO CD3 ZETA LOCUS BY HOMOLOGOUS RECOMBINATION
(= CD3 ZETA SEQUENCE PRESENT IN TARGETING VECTOR)

FIG.—9A

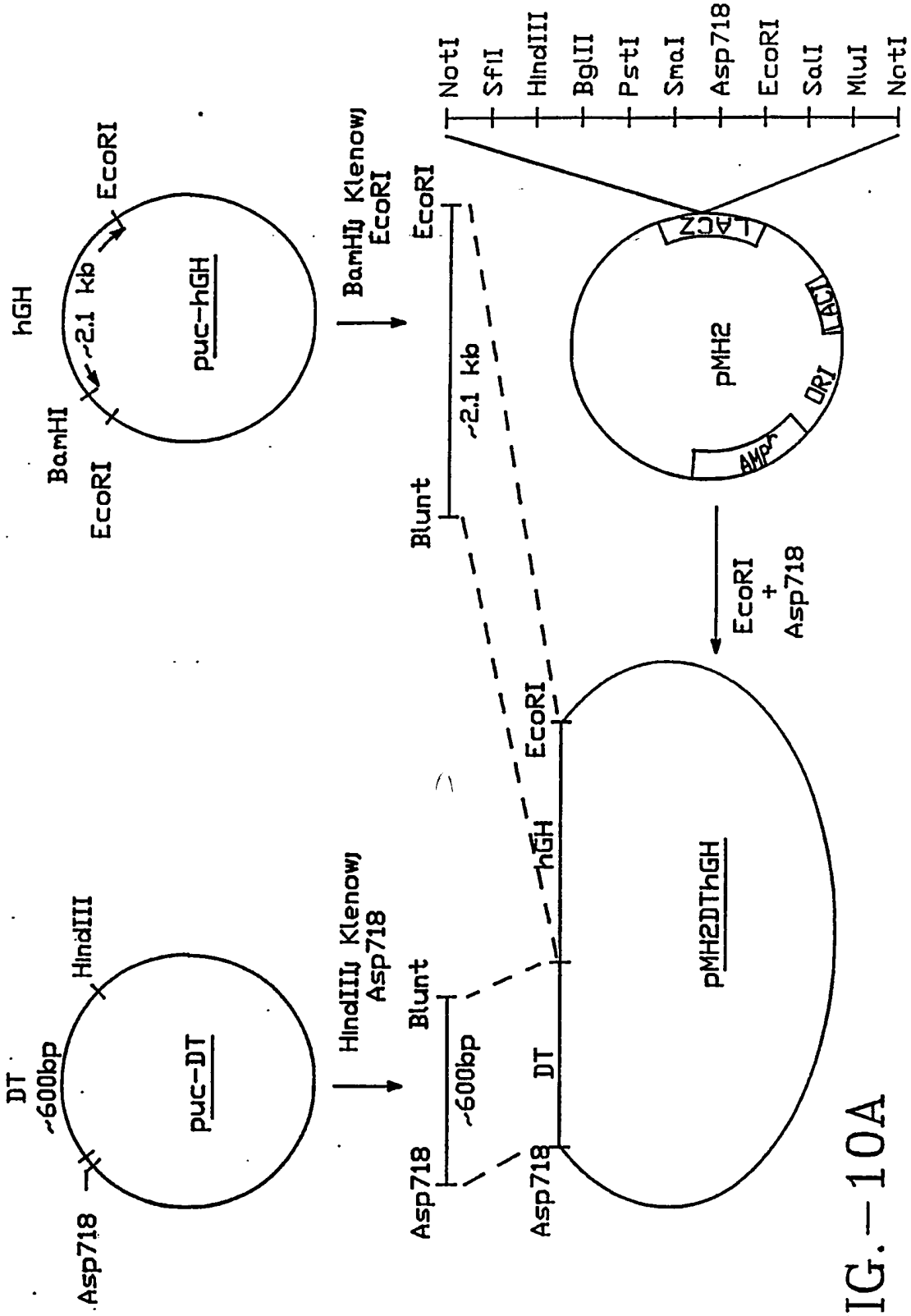

puc-hGH
hGH
~2.1 kb
EcoRI
BamHI
EcoRI
BamHI Klenow
EcoRI
EcoRI
Blunt
~2.1 kb
NotI
SfiI
HindIII
BglII
PstI
SmaI
Asp718
EcoRI
SalI
MluI
NotI
pMH2
LACZ
LACI
ORI
AMP
EcoRI
+
Asp718
puc-DT
DT
~600bp
Asp718
HindIII Klenow
Asp718
Blunt
~600bp
DT
hGH
EcoRI
Asp718
pMH2DThGH

FIG.—10A

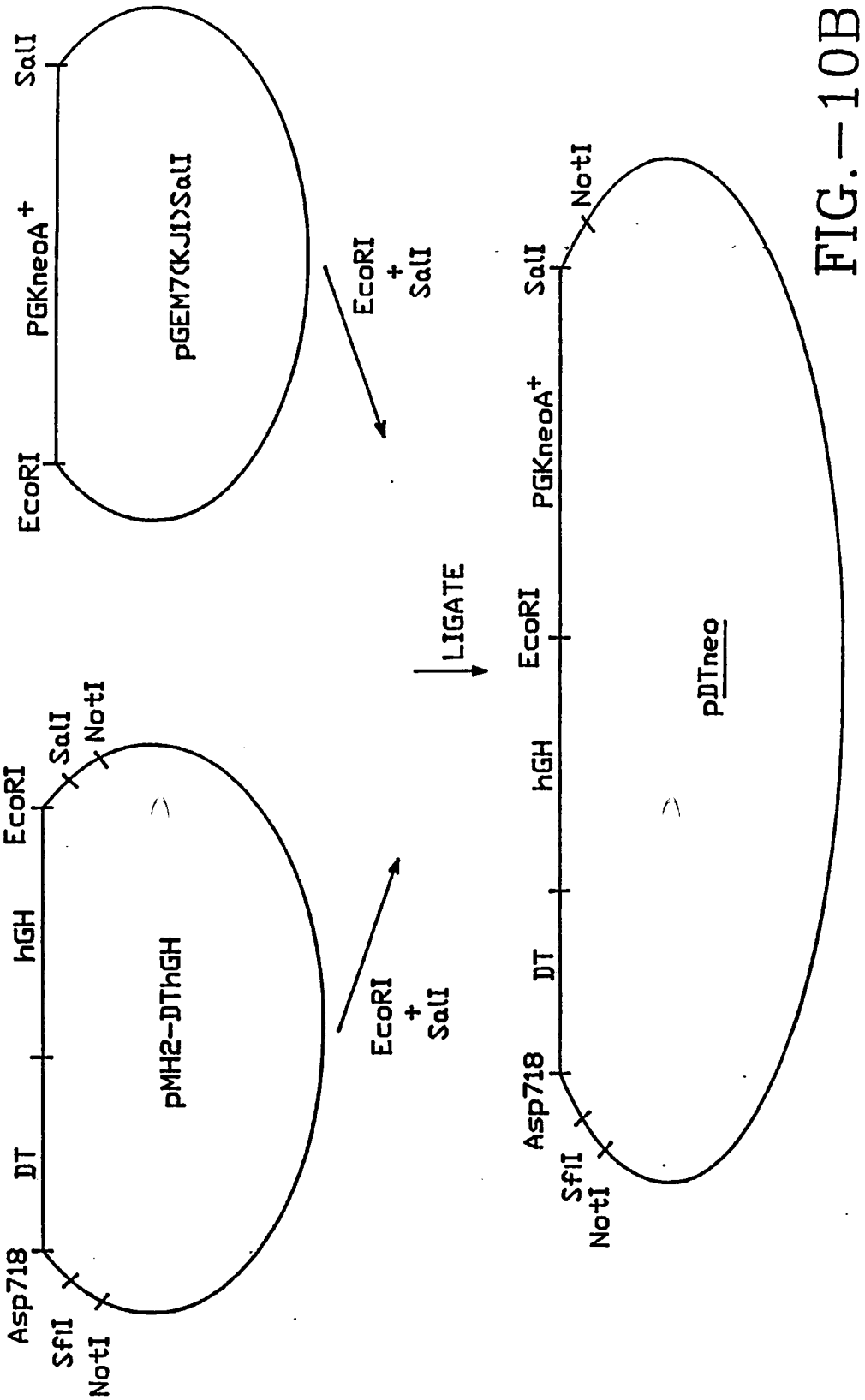

SalI
PGKneoA+
pGEM7(KJ1)SalI
EcoRI
EcoRI
+
SalI
LIGATE
NotI
SalI
PGKneoA+
EcoRI
pDTneo
hGH
DT
Asp718
SfiI
NotI
EcoRI
SalI
NotI
hGH
pMH2-DThGH
DT
Asp718
SfiI
NotI
EcoRI
+
SalI

FIG.-10B

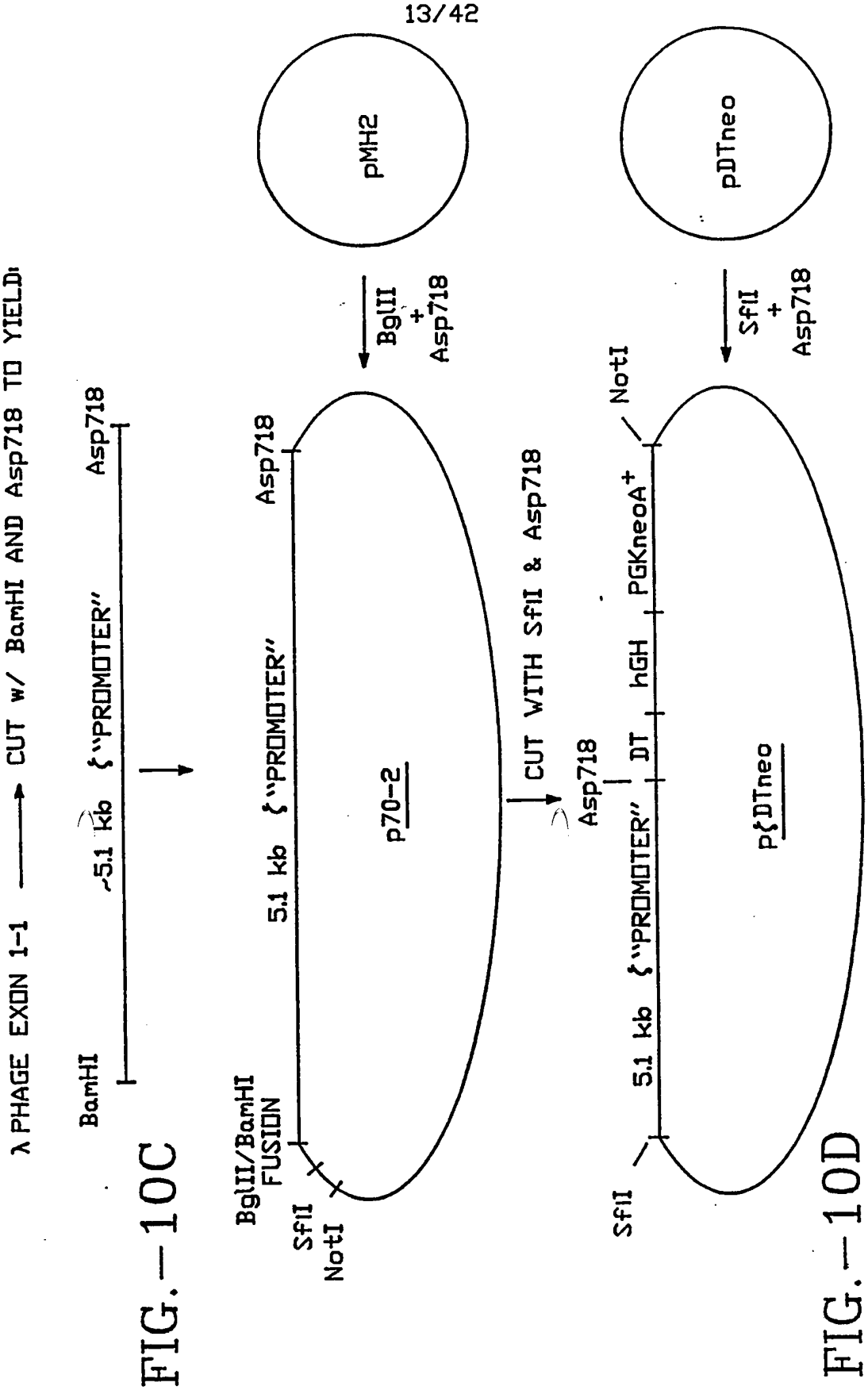


FIG.—10C

FIG.—10D

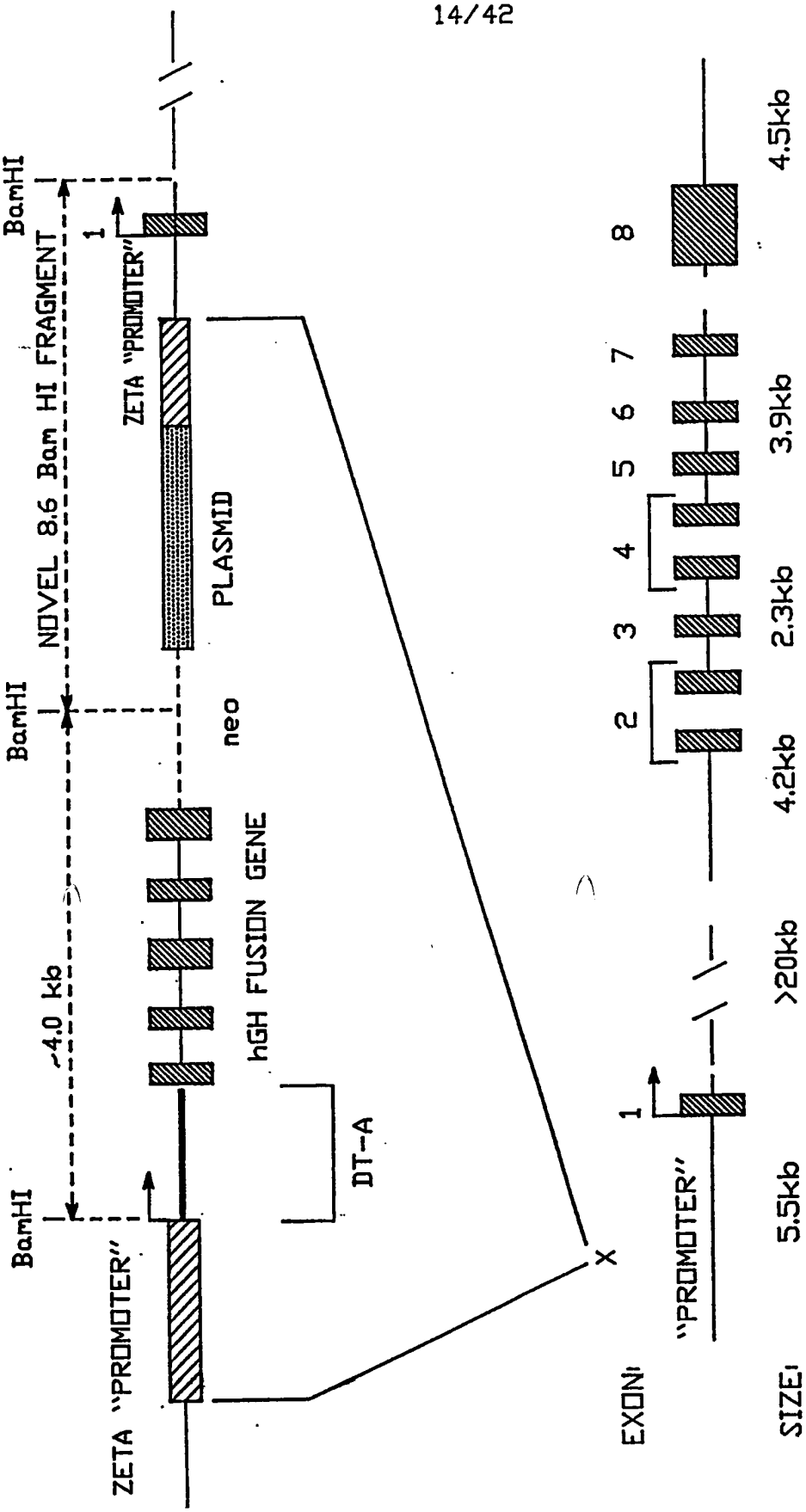

BamHI
NOVEL 8.6 Bam HI FRAGMENT
BamHI
~4.0 kb
BamHI
ZETA "PROMOTER"
1
ZETA "PROMOTER"
PLASMID
neo
hGH FUSION GENE
DT-A
X
EXON:
SIZE:
"PROMOTER"
1
>20kb
5.5kb
2
3
4
4.2kb
2.3kb
5
6
7
3.9kb
8
4.5kb
CD3 ZETA GENE

FIG.—11

CONSTRUCTION OF p4.2 ζ A⁻ 2.3 TK TARGETING VECTOR

1. CD3 ζ BamHI FRAGMENTS 4.2kb AND 2.3kb SUBCLONED INTO pUC18 AT THE BamHI SITE.

2. pUC18 w/ 2.3kb WAS PARTIALLY DIGESTED WITH BamHI, FILLED IN WITH Klenow AND RELIGATED TO ELIMINATE THE 5' BamHI SITE OF EXON 2.

3. A SYNTHETIC OLIGONUCLEOTIDE LINKER WAS PREPARED TO INTRODUCE STOP CODONS IN ALL 3 READING FRAMES:

   5'-AATTACTGCAGCTAGCTAGCTAGT
   TGACGTCGATCGATCGATCATTAA-5'
   pst I

   ← CONTAINS ARMS THAT CAN LIGATE TO EcoRI STICKY ARMS, BUT NOT REGENERATE THE EcoRI SITE AT EITHER END.

4. THE 4.2kb BamHI CD3 ζ FRAGMENT WAS CUT OUT OF pUC18 USING HindIII AND EcoRI PGK neo (POLY A⁻ VERSION) WAS CUT OUT OF pGEM7(FJ1) SalI POLY A⁻ USING EcoRI AND SalI. A FOUR-PART LIGATION WAS PERFORMED AMONG THESE 2 FRAGMENTS PLUS THE SYNTHETIC LINKER AND pMH5:

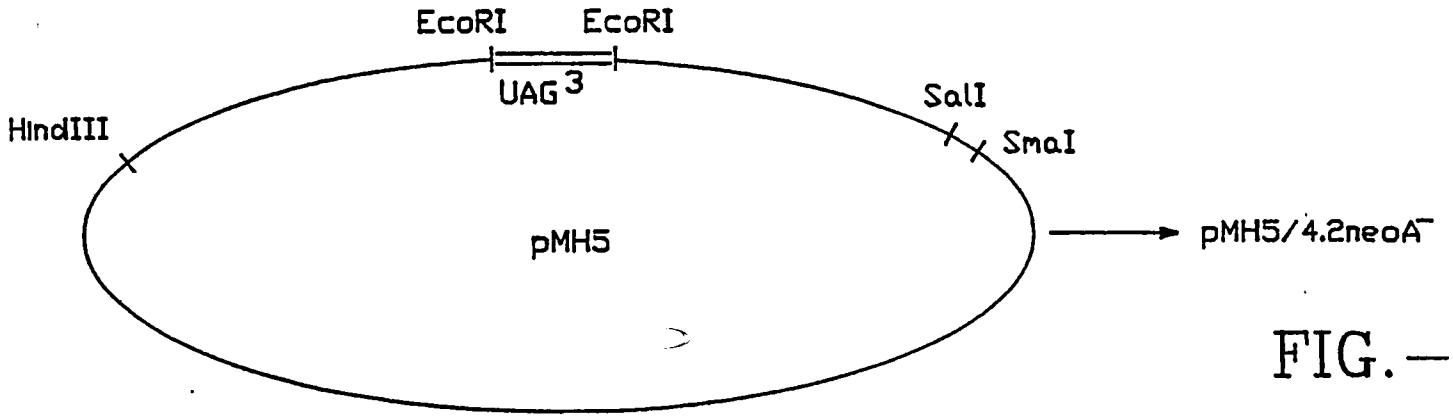
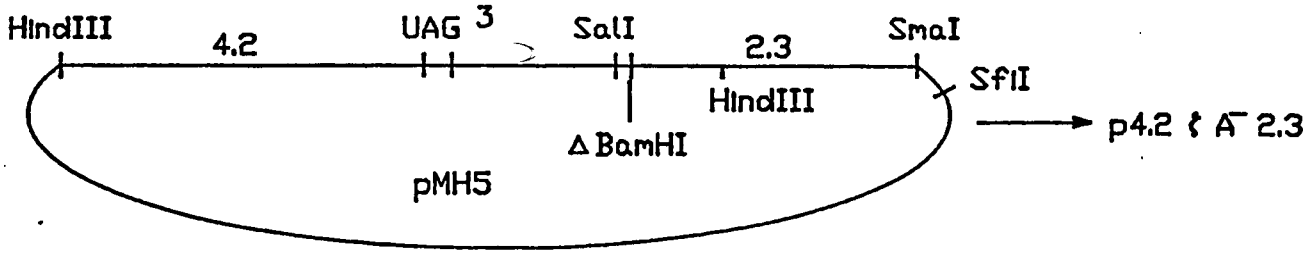
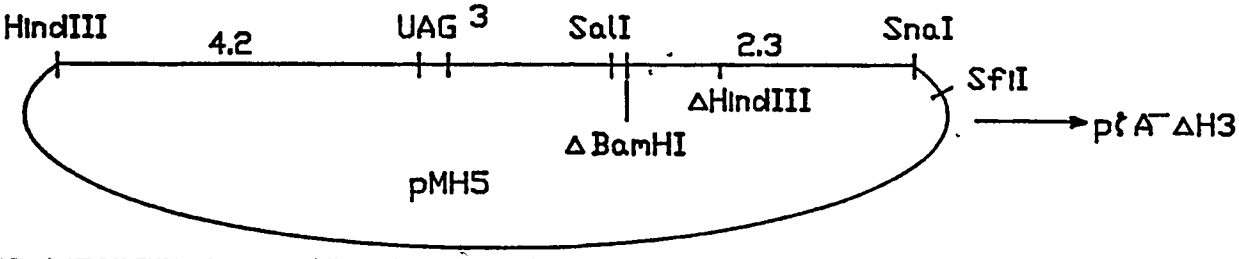


FIG.—12

5. THE 2.3kb CD3 ζ BamHI FRAGMENT WAS CUT OUT OF pUC18 WITH salI AND SmaI AND LIGATED INTO THE SalI AND SmaI SITES OF pUH5/4.2neoA⁻ (STEP 4):



6. p4.2 ζ A⁻2.3 WAS PARTIALY DIGESTED WITH HindIII AND BLUNT ENDED WITH Klenow FOLLOWED BY RELIGATION. TRANSFORMENTS WERE SCREENED FOR THOSE THAT HAD LOST THE HindIII SITE WITHIN EXON 2, LOCATED APPROXIMATELY 800bp DOWNSTREAM OF THE FORMER BamHI SITE IN EXON 2:



pMCITK (IN pKS VECTOR) WAS CUT OUT WITH BamHI AND HindIII AND SUBCLONED INTO pMH2 AT THE BglII AND HindIII SITES:

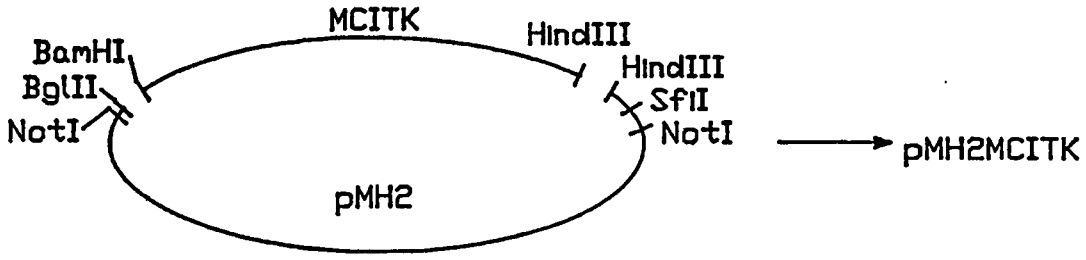
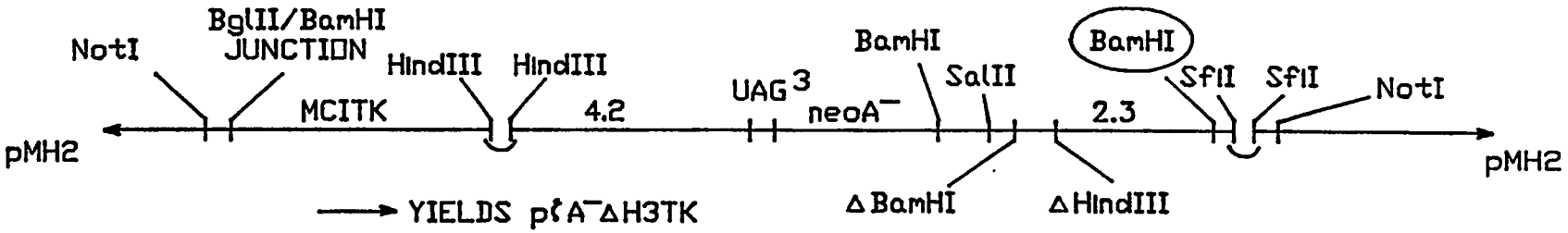


FIG.—12
(CONTINUED)

8. p4.2 ʃA⁻2.3 ΔH3 (STEP 6) DIGESTED WITH HindIII AND SfiI AND THE INSERT SUBCLONED INTO THE SAME SITES OF pMH2MCITK (STEP 7):



9. pʃA⁻ΔH3TK WAS PARTIALLY DIGESTED WITH BamHI, BLUNT-ENDED WITH Klenow, AND SELF-LIGATED TO YIELD pʃA⁻ΔTKf.
(SAME AS 8, WITH THE CIRCLED BamHI SITE DESTROYED BY BLUNT-ENDED LIGATION)

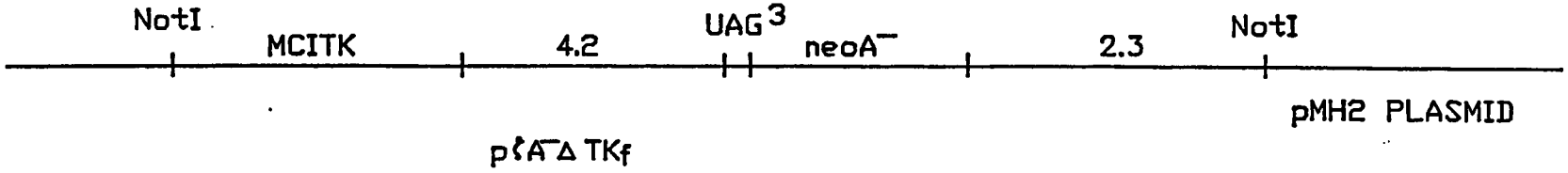


FIG.—12
(CONTINUED)

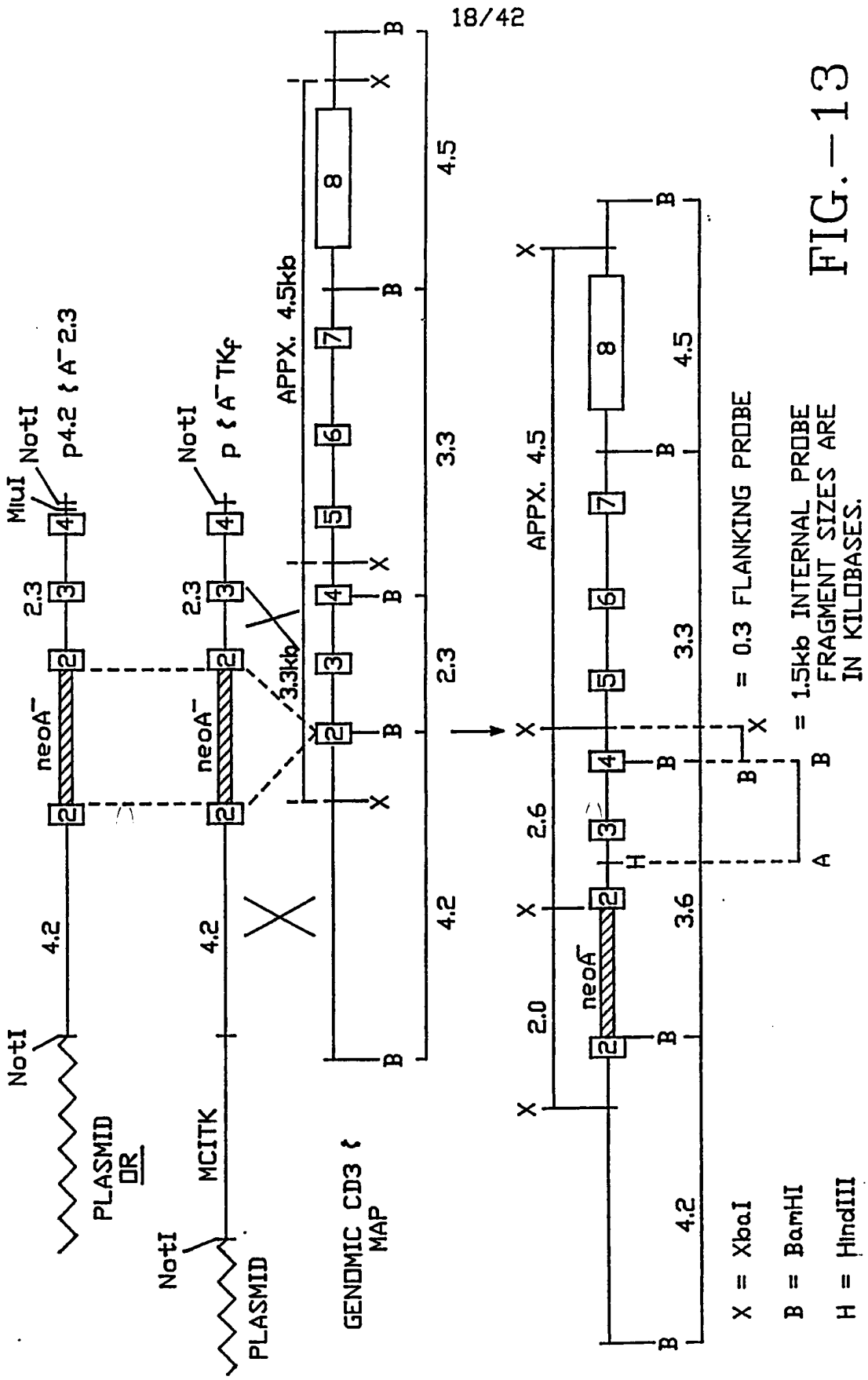

MluI
NotI
p4.2 ζ A⁻2.3
NotI
p ζ A⁻TKp
APPX. 4.5kb
2.3
neoA⁻
4.2
NotI
PLASMID
OR
MCITK
NotI
PLASMID
GENOMIC CD3 ζ
MAP
3.3kb
4.5
3.3
2.3
4.2
APPX. 4.5
2.6
2.0
3.6
= 0.3 FLANKING PROBE
= 1.5kb INTERNAL PROBE
FRAGMENT SIZES ARE
IN KILOBASES.
X = XbaI
B = BamHI
H = HindIII

FIG.—13

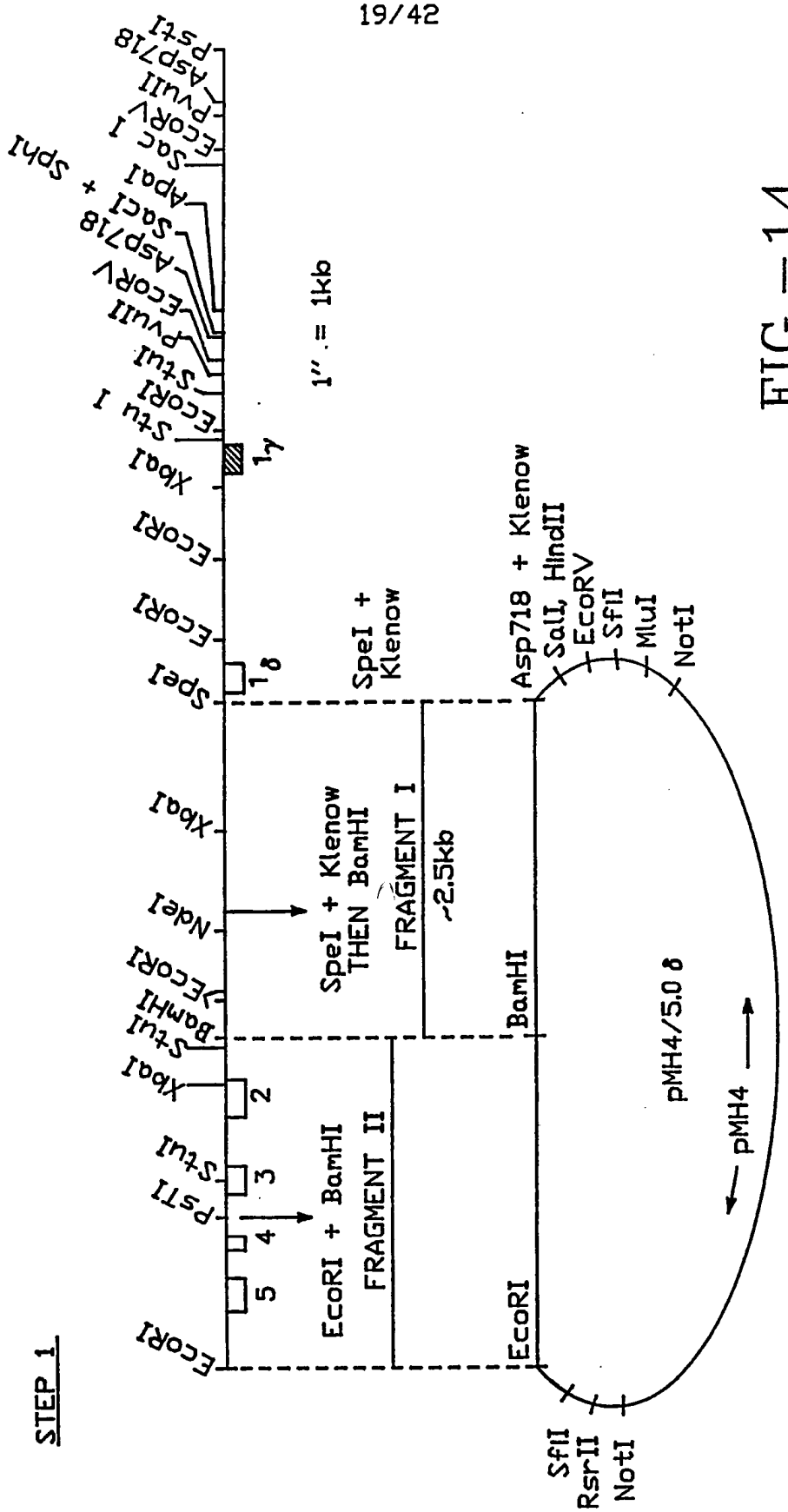

PTM δ
STEP 1
1" = 1kb
1γ
1δ
2
3
4
5
EcoRI
PstI
StuI
XbaI
NdeI
BamHI
EcoRI
SpeI
XbaI
EcoRI
EcoRI
StuI
XbaI
EcoRI
PvuII
EcoRV
Asp718
SacI
ApaI
SacI + SphI
EcoRV
PvuII
StuI
EcoRI
XbaI
StuI I
SpeI + Klenow THEN BamHI
FRAGMENT I
~2.5kb
EcoRI + BamHI
FRAGMENT II
SpeI + Klenow
Asp718 + Klenow
SalI, HindII
EcoRV
SfiI
MluI
NotI
BamHI
EcoRI
pMH4/5.0 δ
pMH4
SfiI
RsrII
NotI

FIG.—14

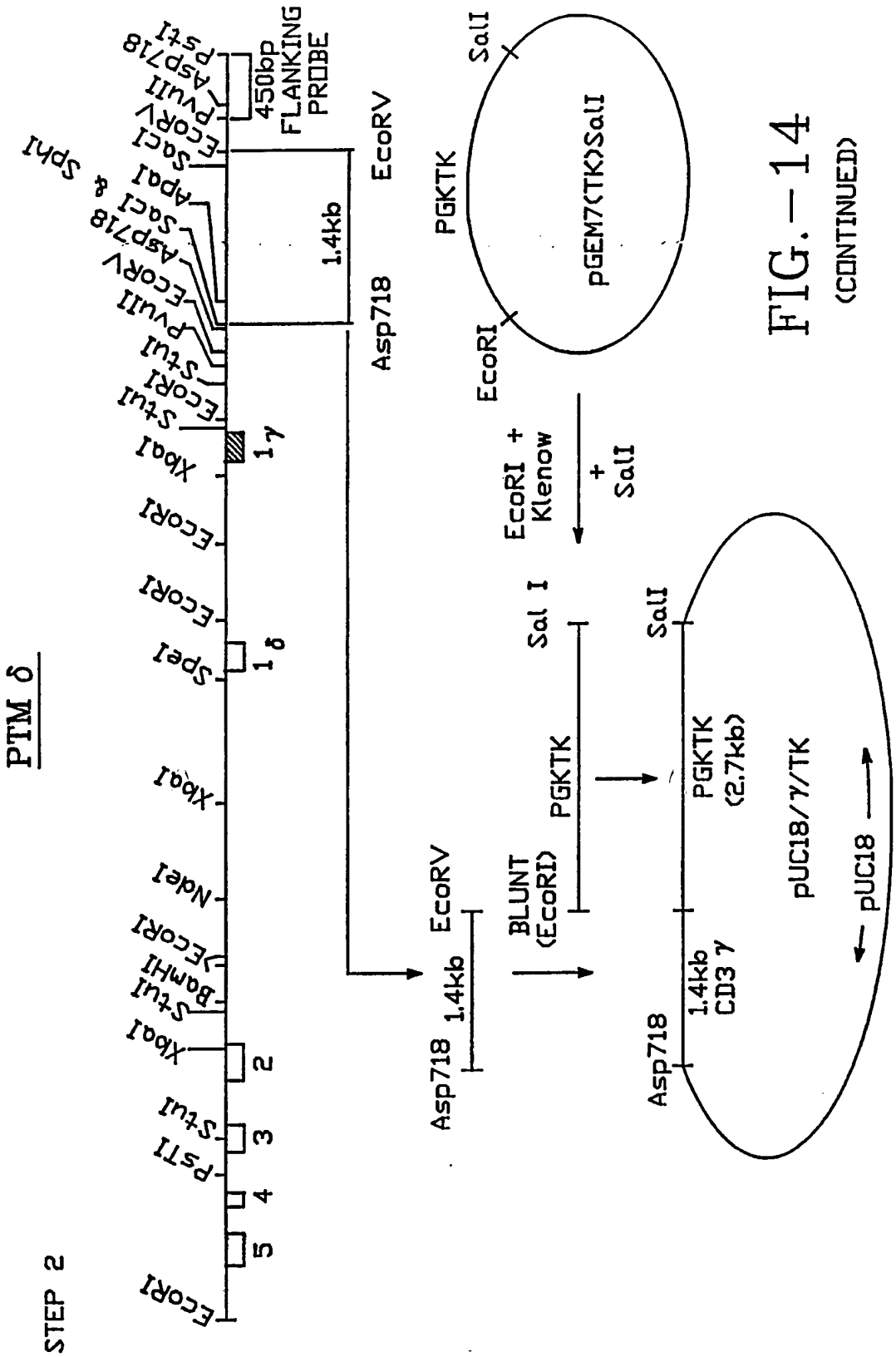


FIG.-14
(CONTINUED)

SUBSTITUTE SHEET

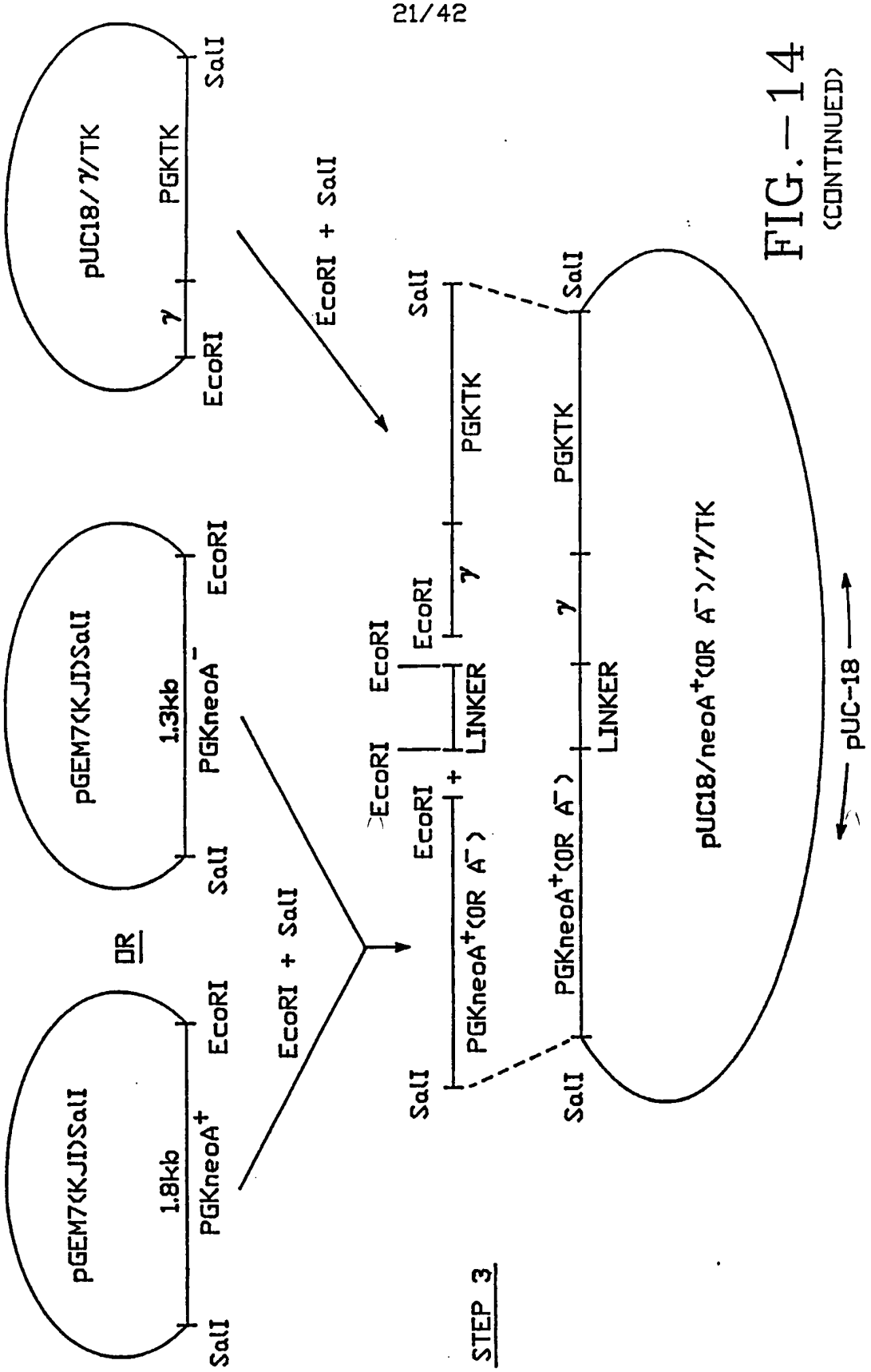


FIG.-14
(CONTINUED)

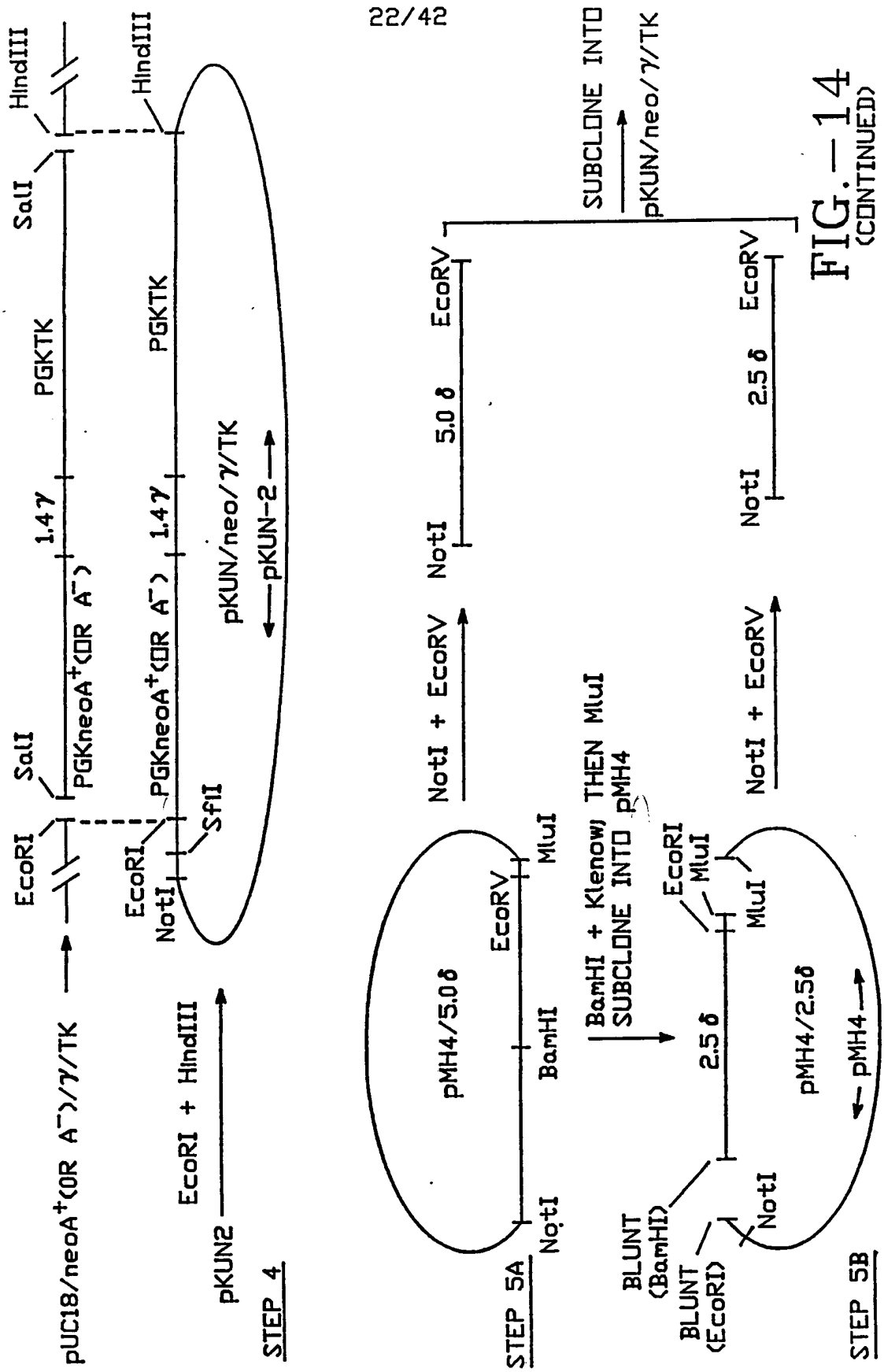


FIG.—14
(CONTINUED)

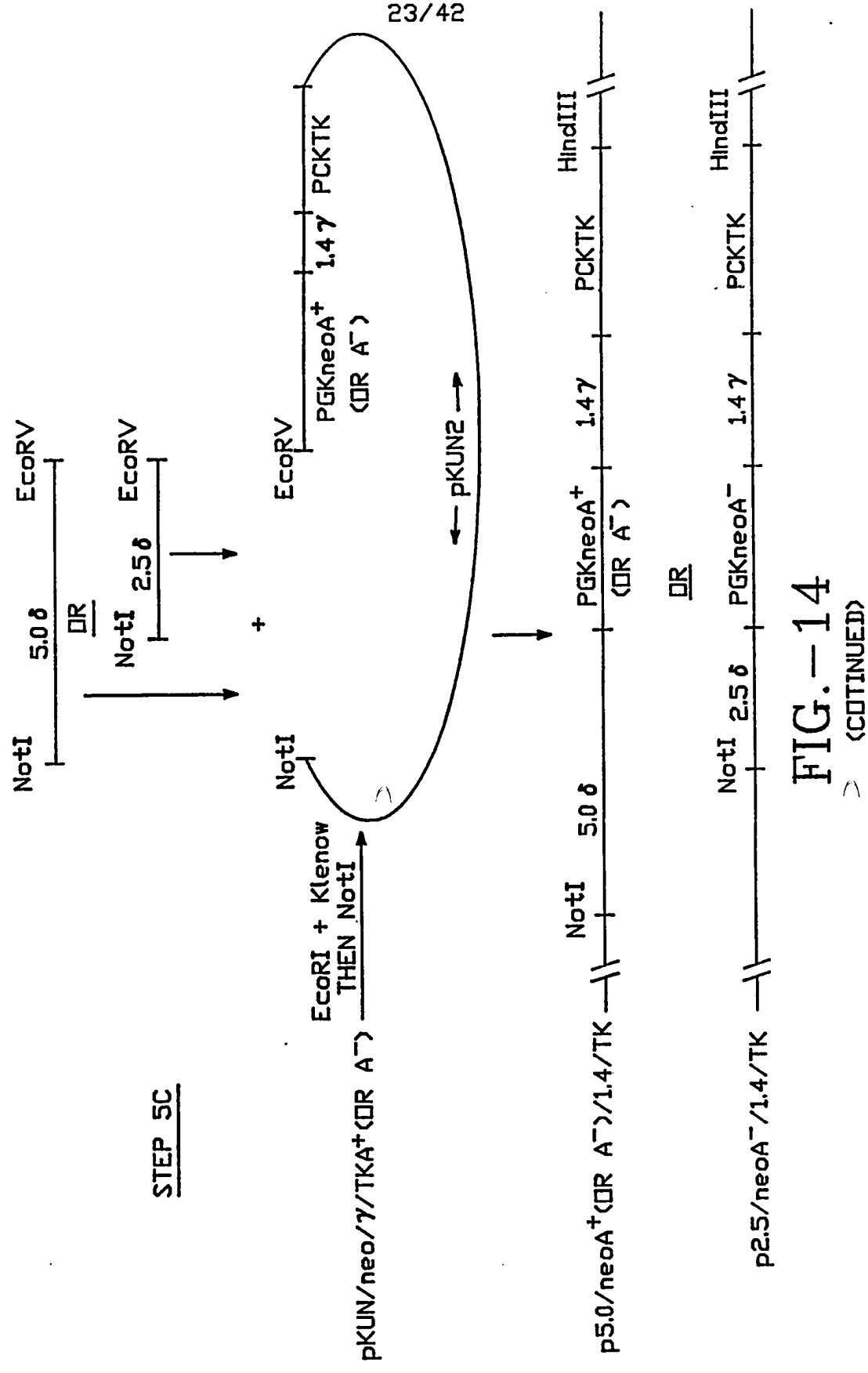


FIG.—14
(CONTINUED)

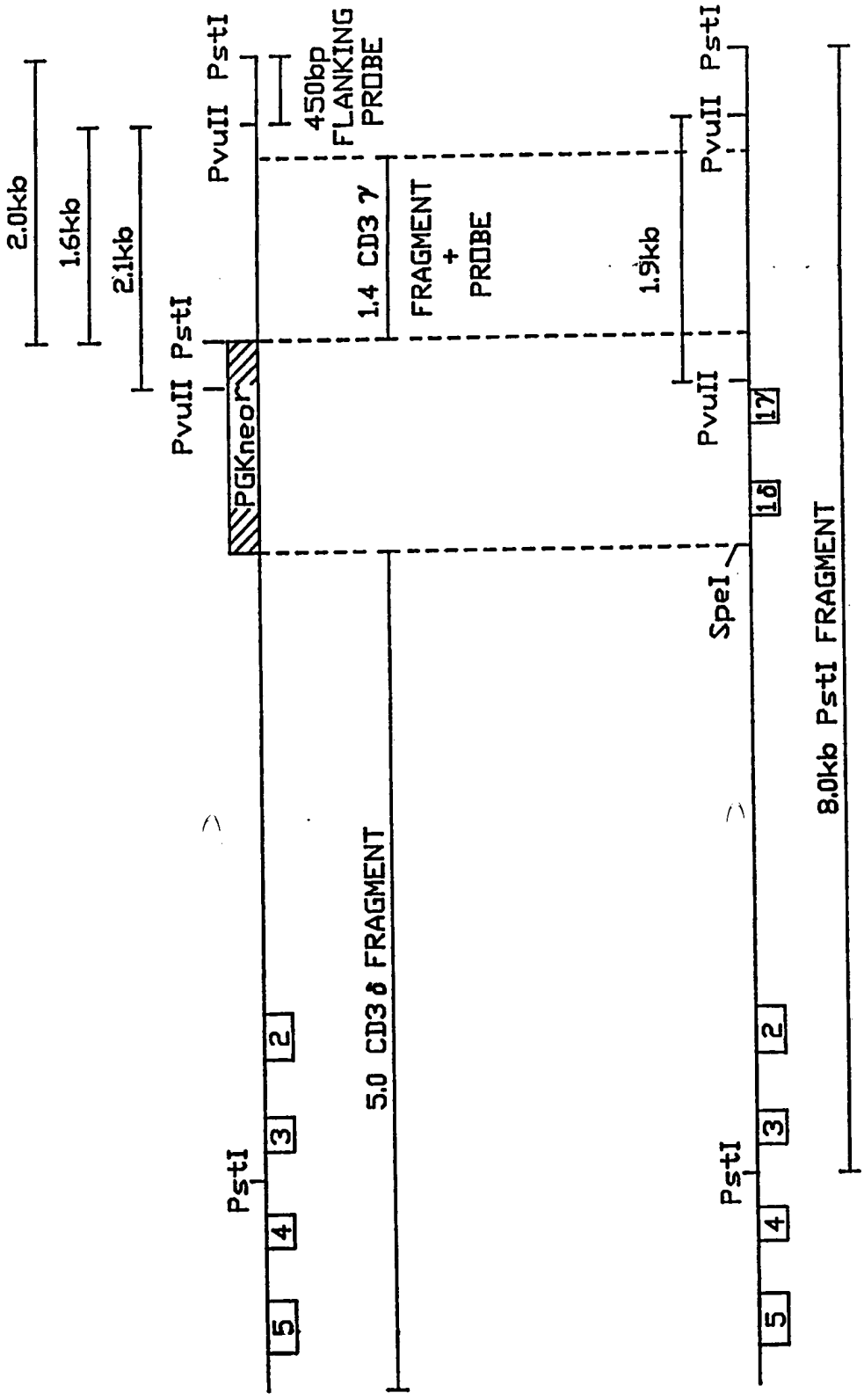
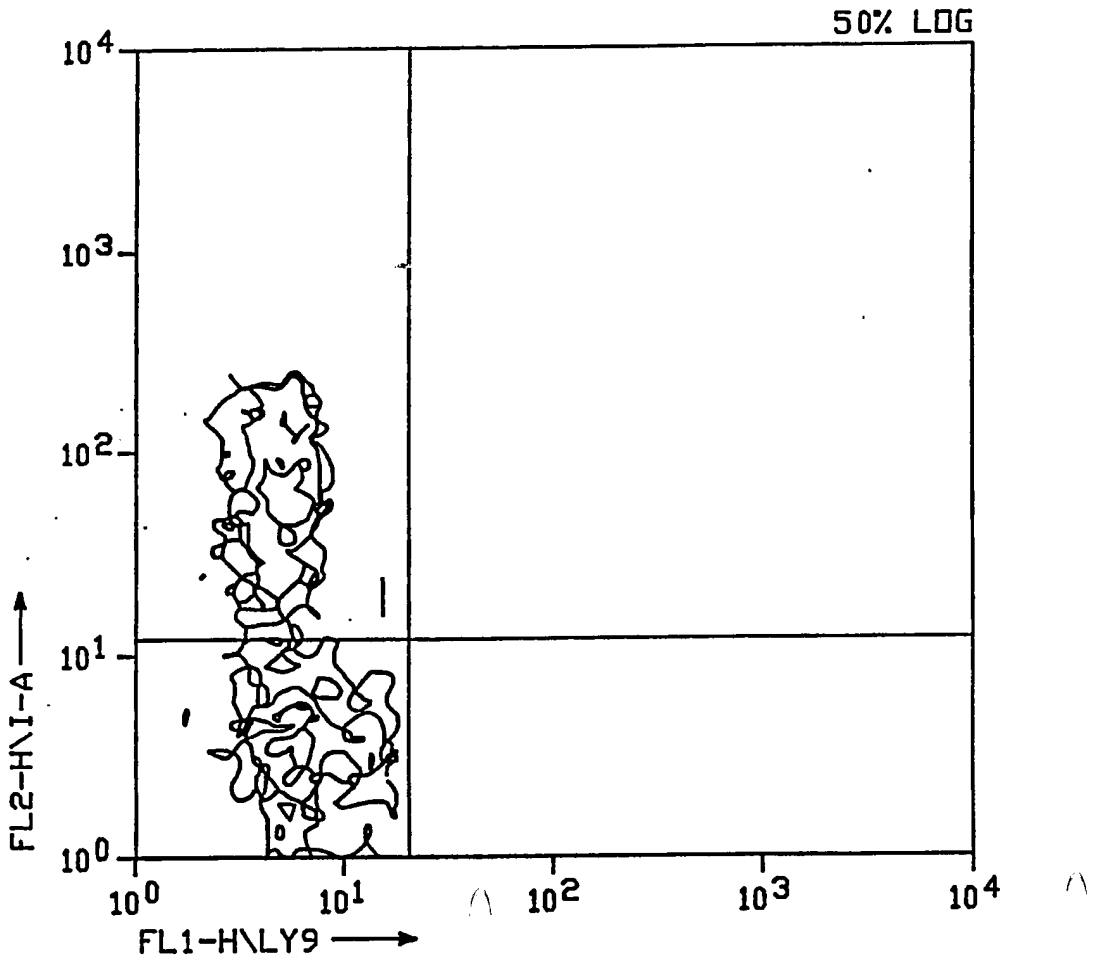

2.0kb
1.6kb
2.1kb
PvuII PstI
450bp FLANKING PROBE
1.4 CD3 γ FRAGMENT + PROBE
1.9kb
PvuII PstI
PvuII
PGKneor
17
16
SpeI
8.0kb PstI FRAGMENT
5.0 CD3 δ FRAGMENT
PstI
2
3
4
5

FIG.—15

SAMPLE 009



QUAD STATS

SAMPLE: B6/I-A/LY9 009

GATE G1= R1

PARAMETERS: FL1-H(LOG),FL2-H(LOG)

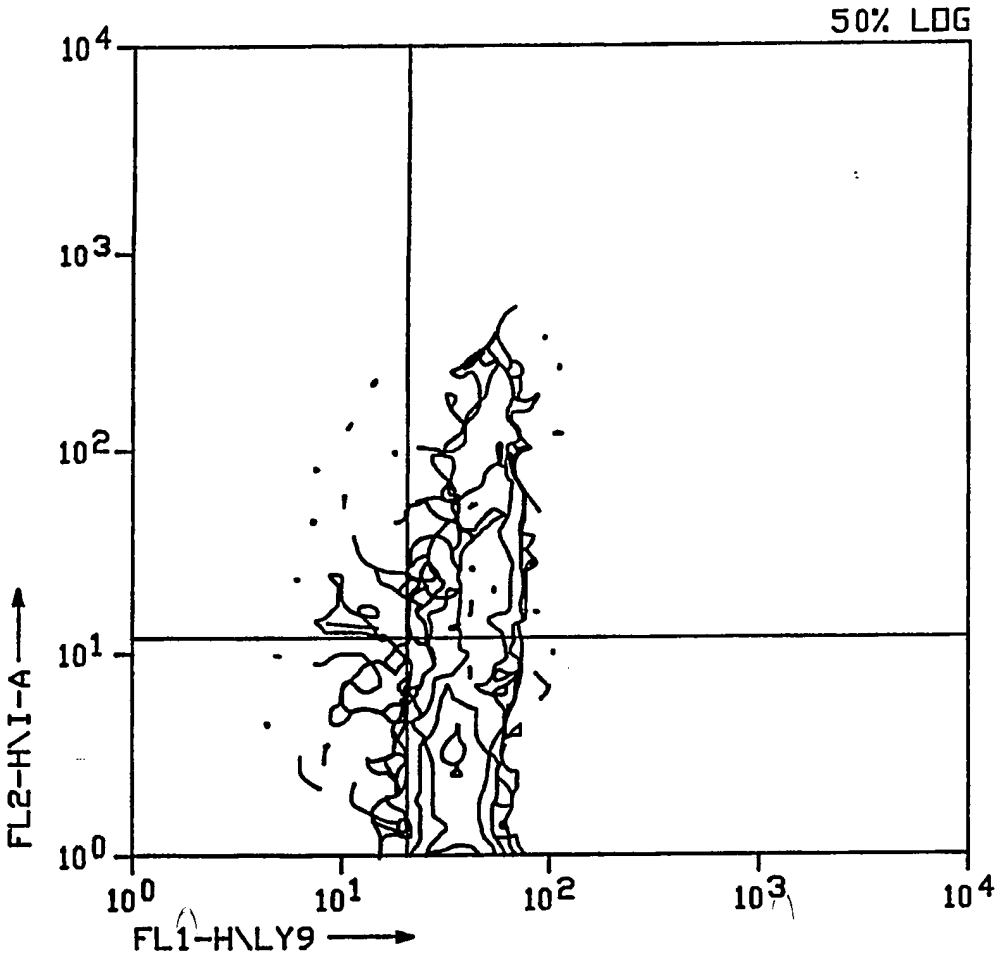
QUAD LOCATION: 20.53,11

TOTAL= 5000 GATED= 1557

QUAD	EVENTS	% GATED	% TOTAL	Xmean	Ymean
1 UL	802	51.51	16.04	4.96	68.48
2 UR	11	0.71	0.22	31.51	41.35
3 LL	742	47.66	14.04	7.16	3.51
4 LR	2	0.13	0.04	24.58	5.13

FIG.—16A

SAMPLE 010



QUAD STATS

SAMPLE: 129/I-A/LY9 010

GATE G1= R1

PARAMETERS: FL1-H(LOG),FL2-H(LOG)

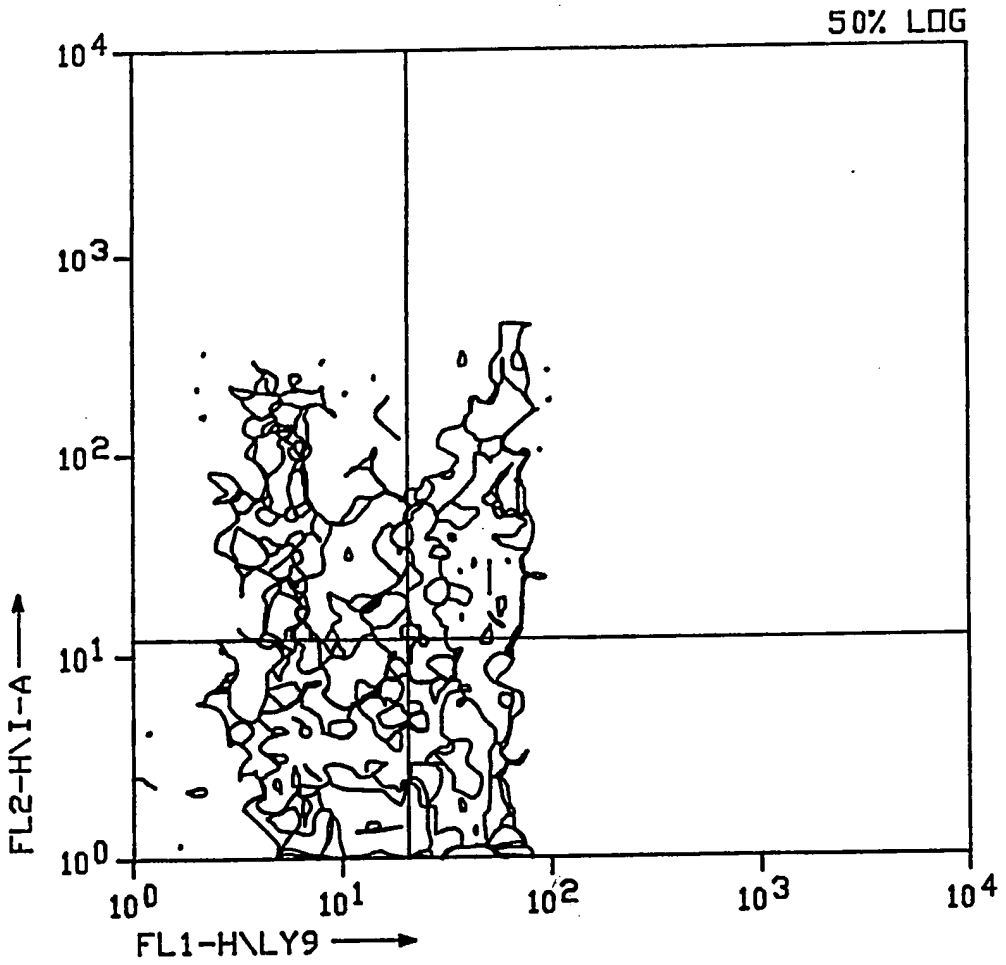
QUAD LOCATION: 20.53,11

TOTAL= 5000 GATED= 2050

QUAD	EVENTS	% GATED	% TOTAL	Xmean	Ymean
1 UL	38	1.85	0.76	12.35	26.61
2 UR	577	28.04	11.54	50.23	41.75
3 LL	66	3.21	1.32	14.02	3.61
4 LR	1377	66.91	27.54	39.41	2.77

FIG.—16B

SAMPLE 011



QUAD STATS

SAMPLE: 027/I-A/LY9 011

GATE G1= R1

PARAMETERS: FL1-H(LOG),FL2-H(LOG)

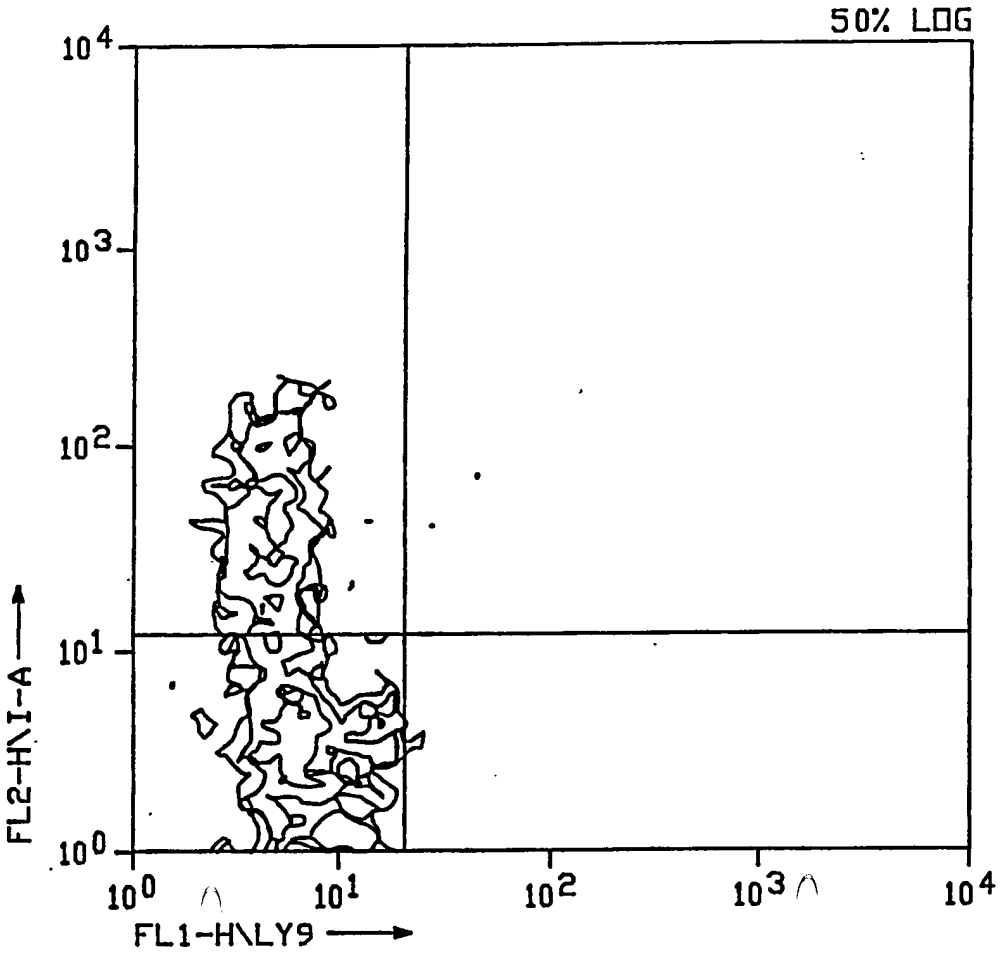
QUAD LOCATION: 20.53,11

TOTAL= 5000 GATED= 1609

QUAD	EVENTS	% GATED	% TOTAL	Xmean	Ymean
1 UL	223	13.86	4.46	6.55	52.70
2 UR	393	24.43	7.86	48.00	48.96
3 LL	332	20.63	6.64	7.27	3.46
4 LR	661	41.08	13.22	39.42	2.76

FIG.—16C

SAMPLE 013



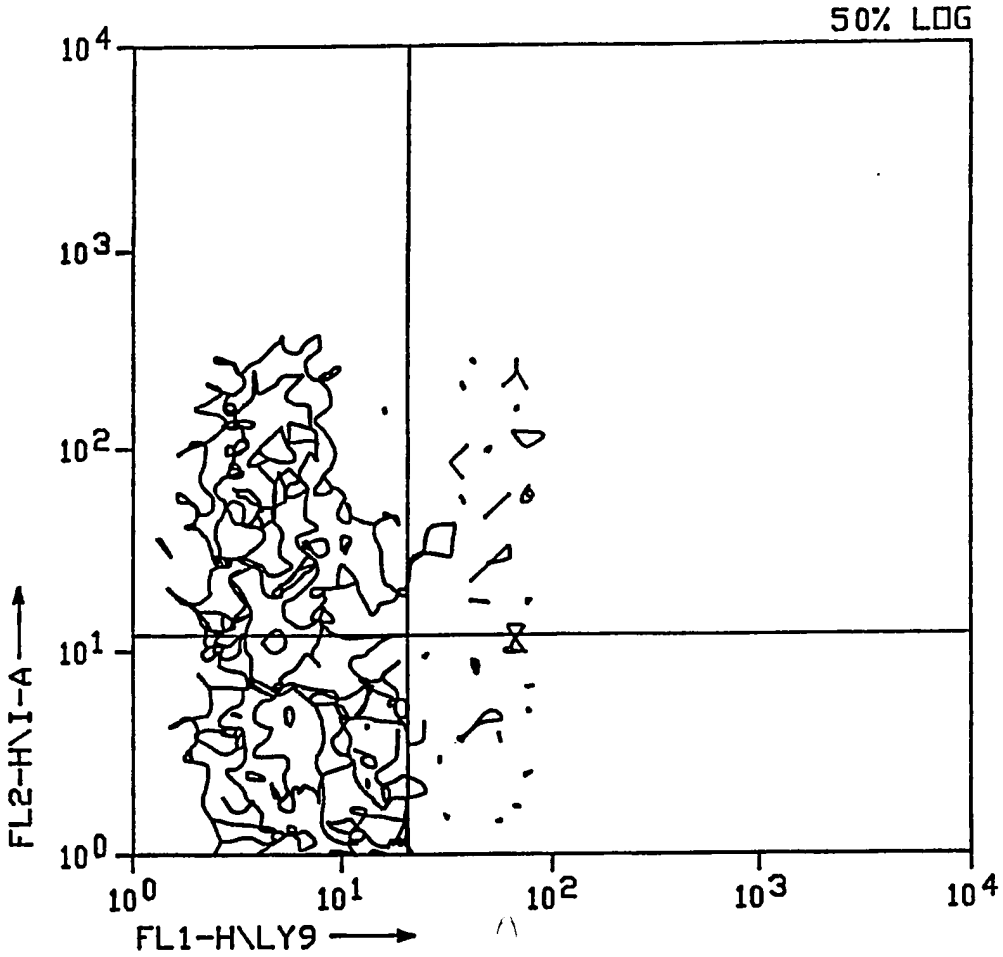
QUAD STATS

SAMPLE: DT061 013
GATE G1= R1
PARAMETERS: FL1-H(LOG),FL2-H(LOG)
QUAD LOCATION: 20.53,11
TOTAL= 5000 GATED= 1856

QUAD	EVENTS	% GATED	% TOTAL	Xmean	Ymean
1 UL	826	44.50	16.52	4.78	47.34
2 UR	38	1.62	0.60	45.58	40.77
3 LL	974	52.48	19.48	6.67	3.51
4 LR	26	1.40	0.52	33.65	3.08

FIG.—16D

SAMPLE 014



QUAD STATS

SAMPLE: DT062 014

GATE G1= R1

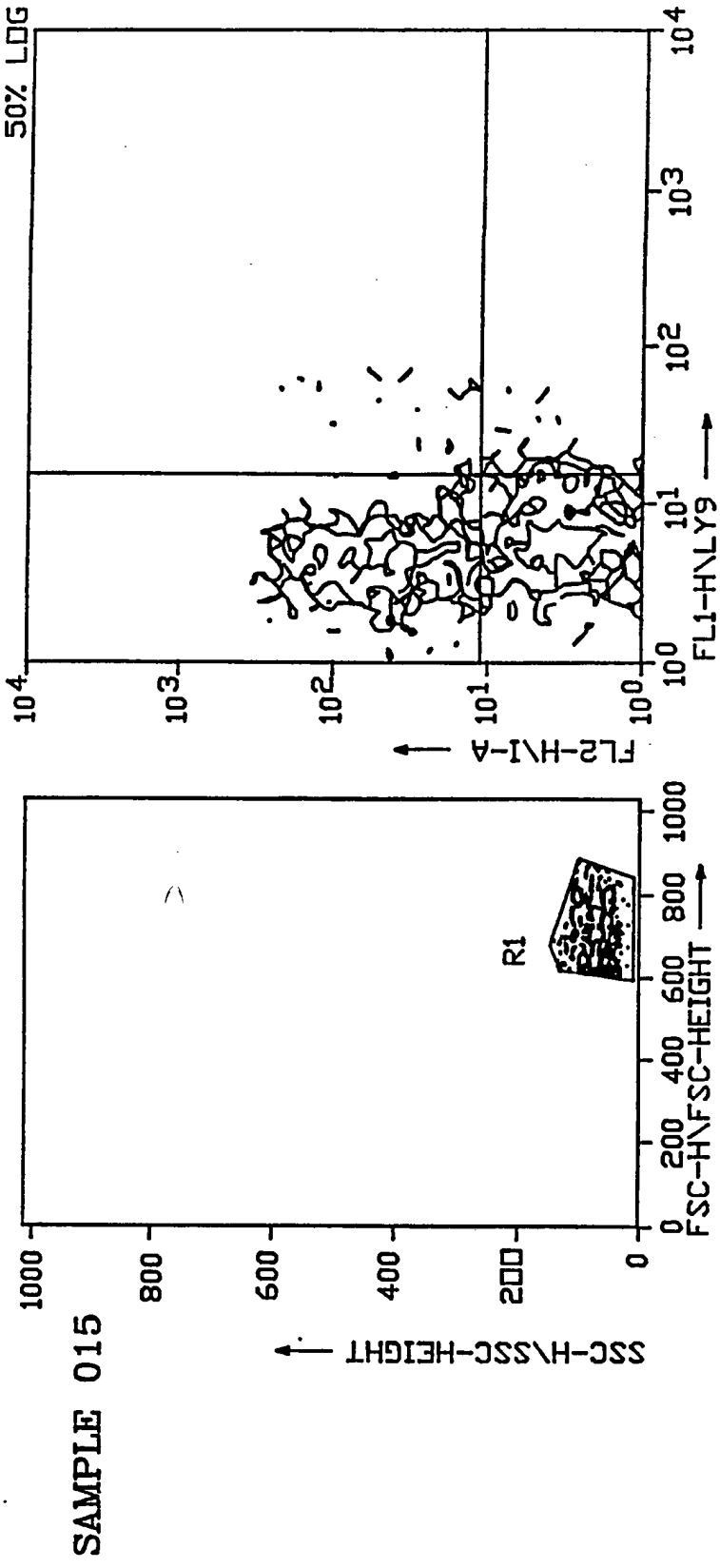
PARAMETERS: FL1-H(LOG),FL2-H(LOG)

QUAD LOCATION: 20.53,11

TOTAL= 5000 GATED= 1804

QUAD	EVENTS	% GATED	% TOTAL	Xmean	Ymean
1 UL	794	44.01	15.88	4.92	54.50
2 UR	48	2.66	0.96	45.69	57.38
3 LL	934	51.37	18.68	6.65	3.43
4 LR	28	1.55	0.56	42.80	3.73

FIG.—16E



QUAD STATS — SAMPLE: DT063 015

GATE G1= R1

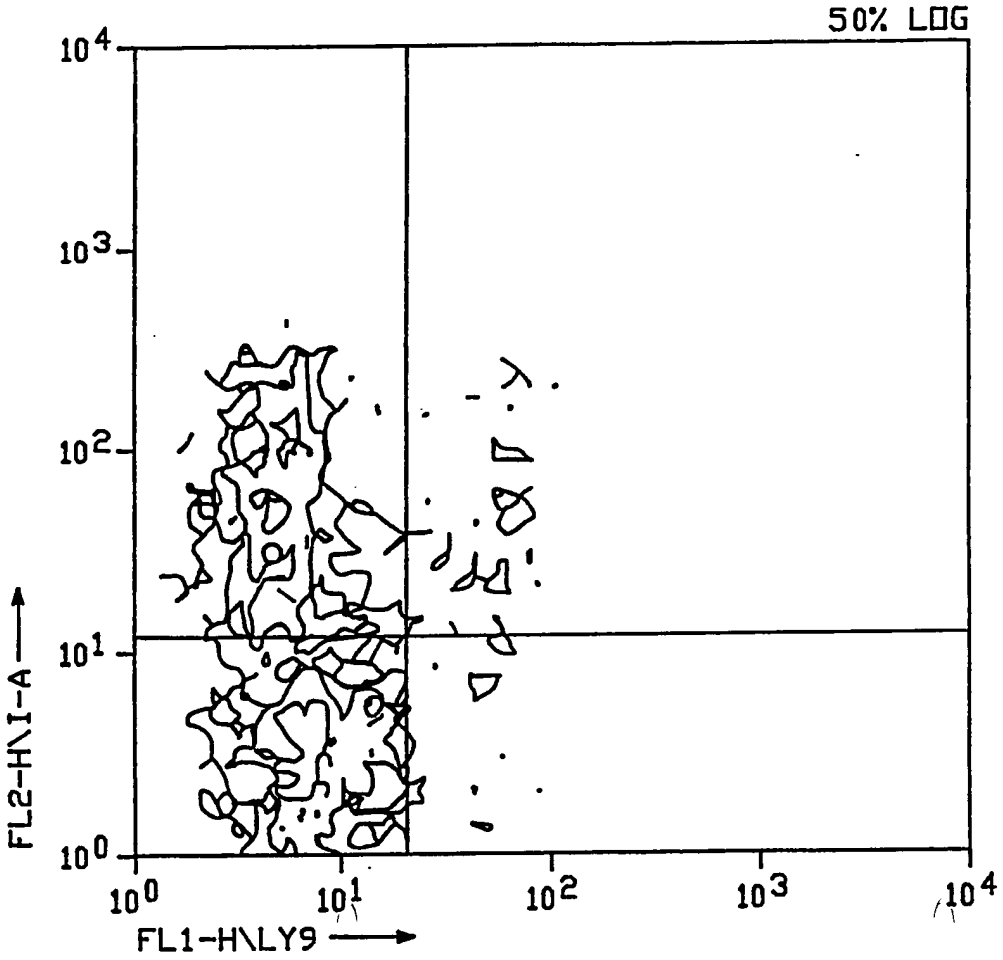
PARAMETERS: FL1-H(LOG),FL2-H(LOG) QUAD LOCATION: 14.33,11

TOTAL= 5000 GATED= 1236

QUAD	EVENTS	% GATED	% TOTAL	Xmean	Ymean
1 UL	573	46.36	11.46	4.54	48.41
2 UR	28	2.27	0.56	34.24	32.69
3 LL	564	45.63	11.28	6.85	3.34
4 LR	71	5.74	1.42	18.42	3.67

FIG.—16F

SAMPLE 016



QUAD STATS

SAMPLE: DT065 016

GATE G1= R1

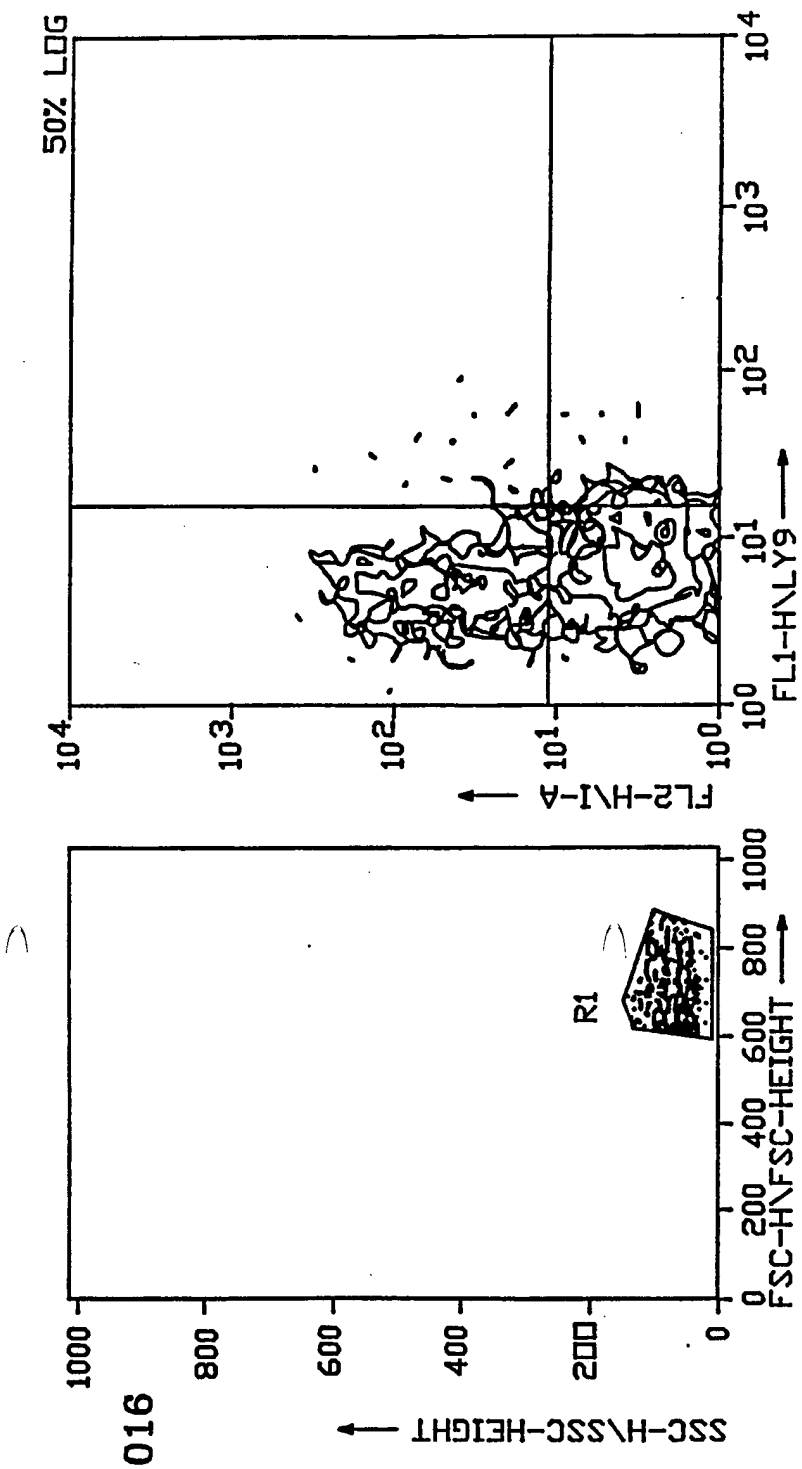
PARAMETERS: FL1-H(LOG),FL2-H(LOG)

QUAD LOCATION: 20.53,11

TOTAL= 5000 GATED= 1613

QUAD	EVENTS	% GATED	% TOTAL	Xmean	Ymean
1 UL	712	44.14	14.24	5.08	57.24
2 UR	66	4.09	1.32	51.48	45.56
3 LL	883	49.91	16.10	7.25	3.31
4 LR	30	1.86	0.60	39.66	4.30

FIG.—16G



QUAD STATS — SAMPLE: DT863 015

GATE G1= R1

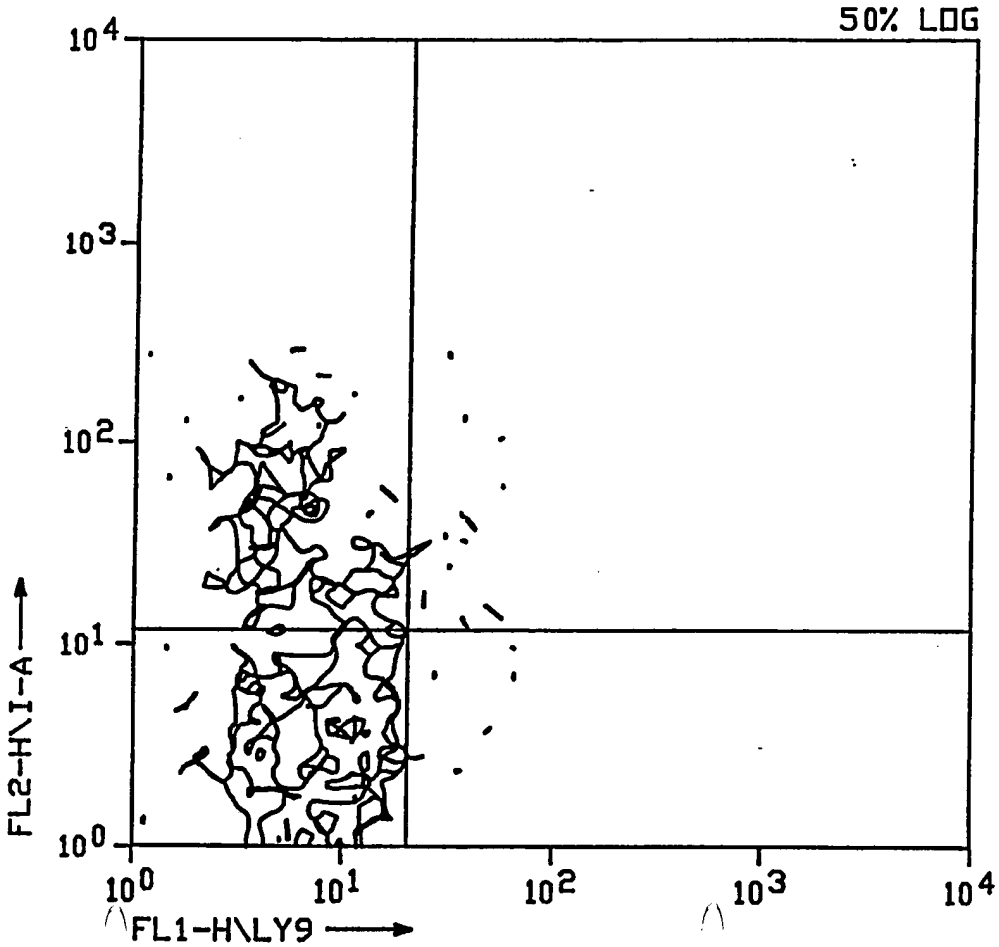
PARAMETERS: FL1-H(LOG),FL2-H(LOG) QUAD LOCATION: 14.33,11

TOTAL= 5000 GATED= 1813

QUAD	EVENTS	% GATED	% TOTAL	Xmean	Ymean
1 UL	706	38.94	14.12	4.88	48.16
2 UR	28	1.54	0.56	24.64	31.58
3 LL	950	52.40	19.00	6.46	3.29
4 LR	129	7.12	2.50	18.07	3.63

FIG.—16H

SAMPLE 018

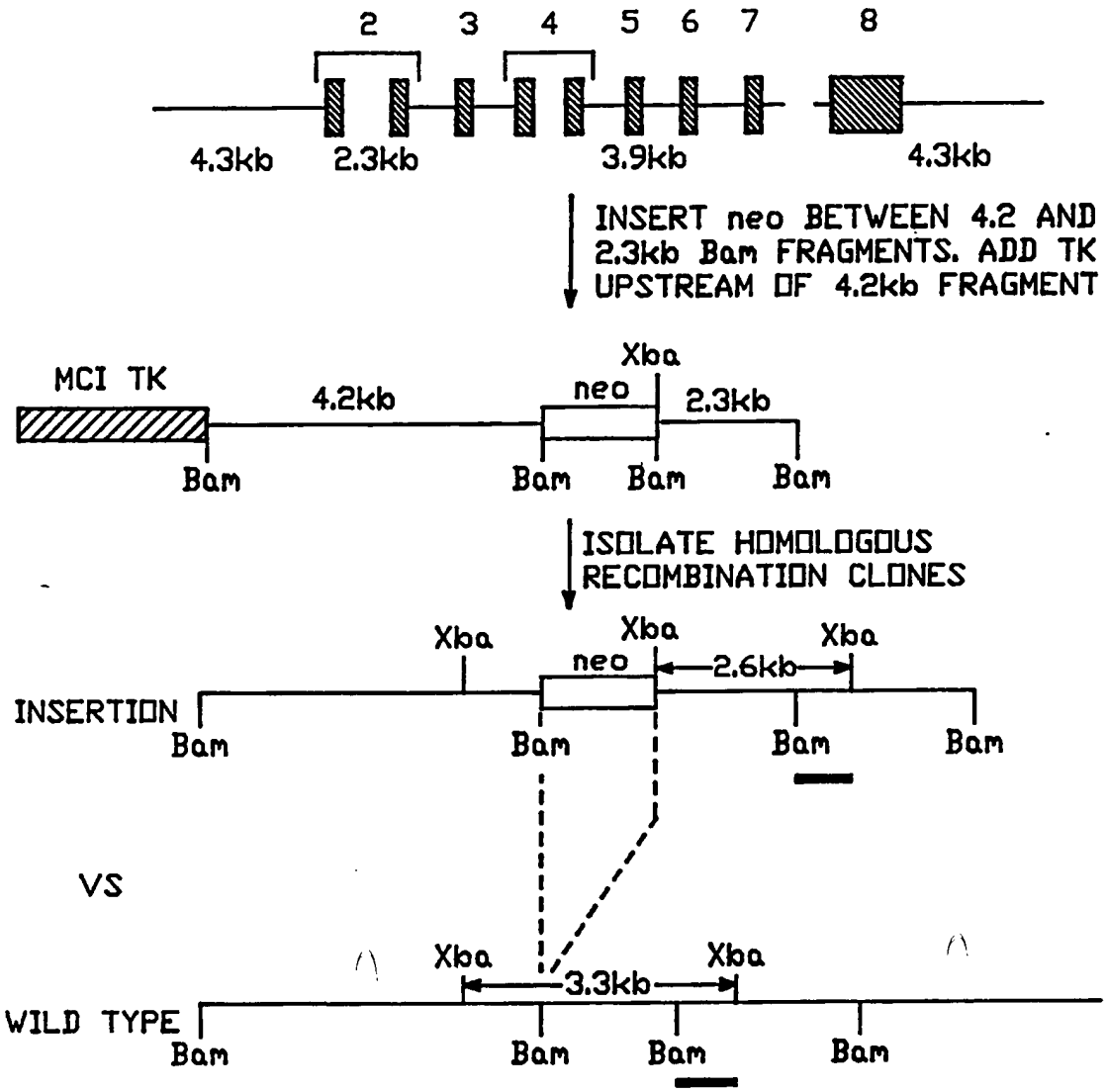


QUAD STATS

SAMPLE: DT067 018
GATE G1= R1
PARAMETERS: FL1-H(LOG),FL2-H(LOG)
QUAD LOCATION: 20.53,11
TOTAL= 5000 GATED= 795

QUAD	EVENTS	% GATED	% TOTAL	Xmean	Ymean
1 UL	197	24.78	3.94	6.39	47.23
2 UR	28	2.52	0.40	36.84	36.91
3 LL	569	71.57	11.38	7.64	3.31
4 LR	9	1.13	0.18	38.46	4.13

FIG.—16 I



CD3 ZETA HOMOLOGOUS RECOMBINATION REPLACEMENT VECTOR. HOMOLOGOUS RECOMBINATION LEADS TO A NOVEL XbaI FRAGMENT OF 2.6kb VS ENDOGENOUS BAND OF 3.3kb. SOLID BAR REPRESENTS DIAGNOSTIC PROBE.

FIG.—17A

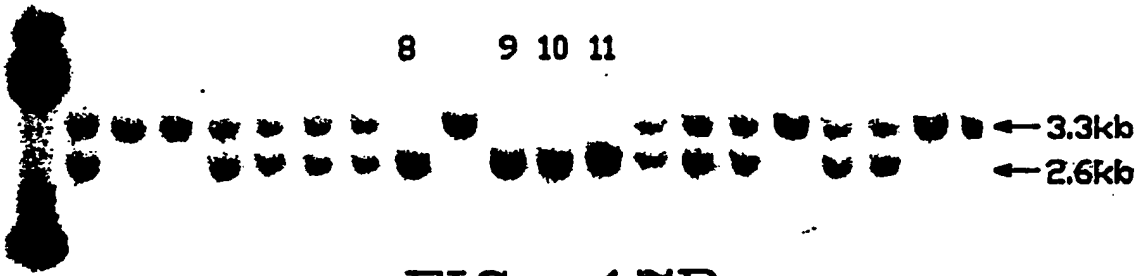


FIG.—17B

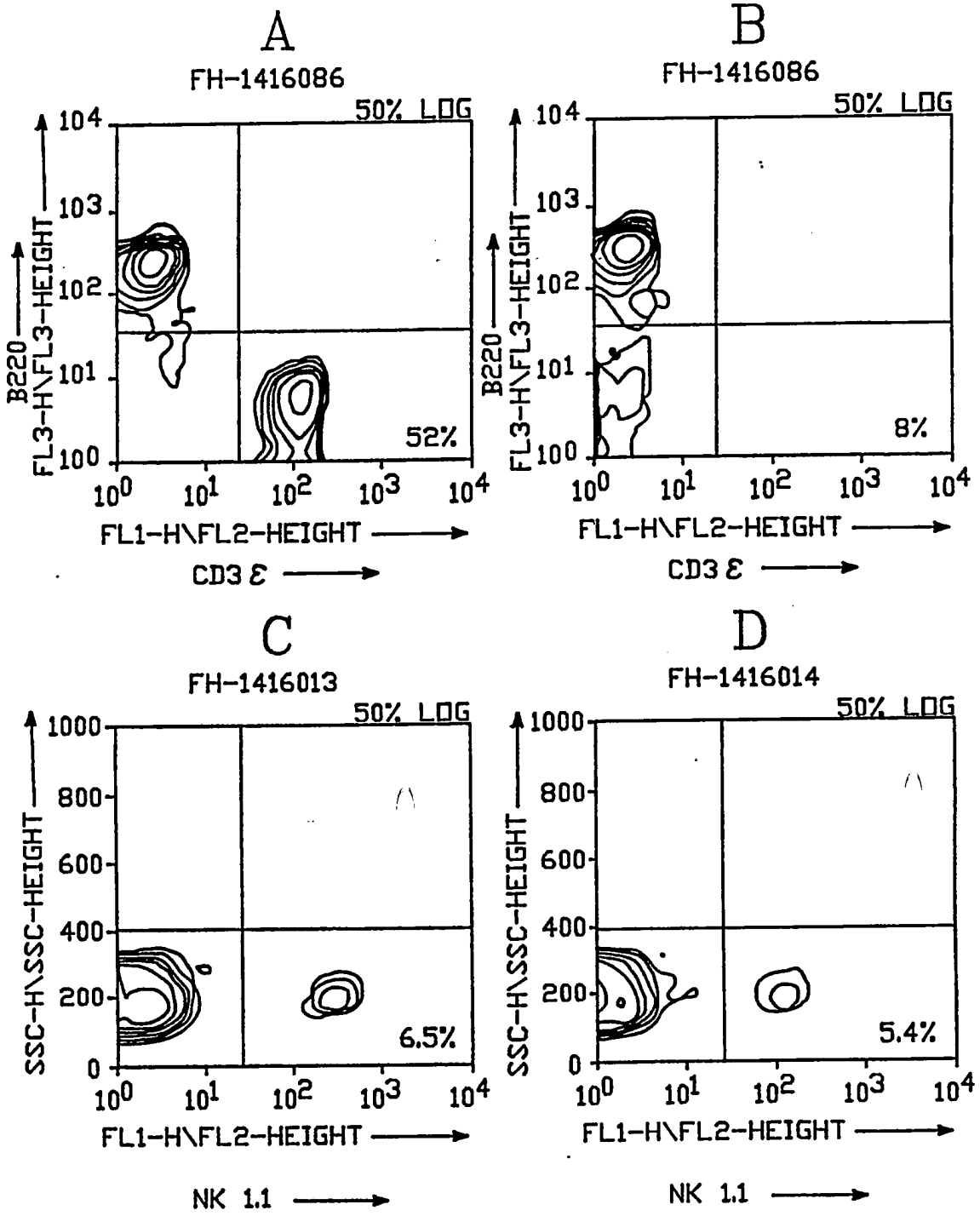

A
FH-1416086
50% LOG
B220
FL3-H\FL3-HEIGHT
52%
FL1-H\FL2-HEIGHT
CD3 ε
B
FH-1416086
50% LOG
8%
C
FH-1416013
50% LOG
SSC-H\SSC-HEIGHT
6.5%
NK 1.1
D
FH-1416014
50% LOG
5.4%

FIG.—18

FIG.—19

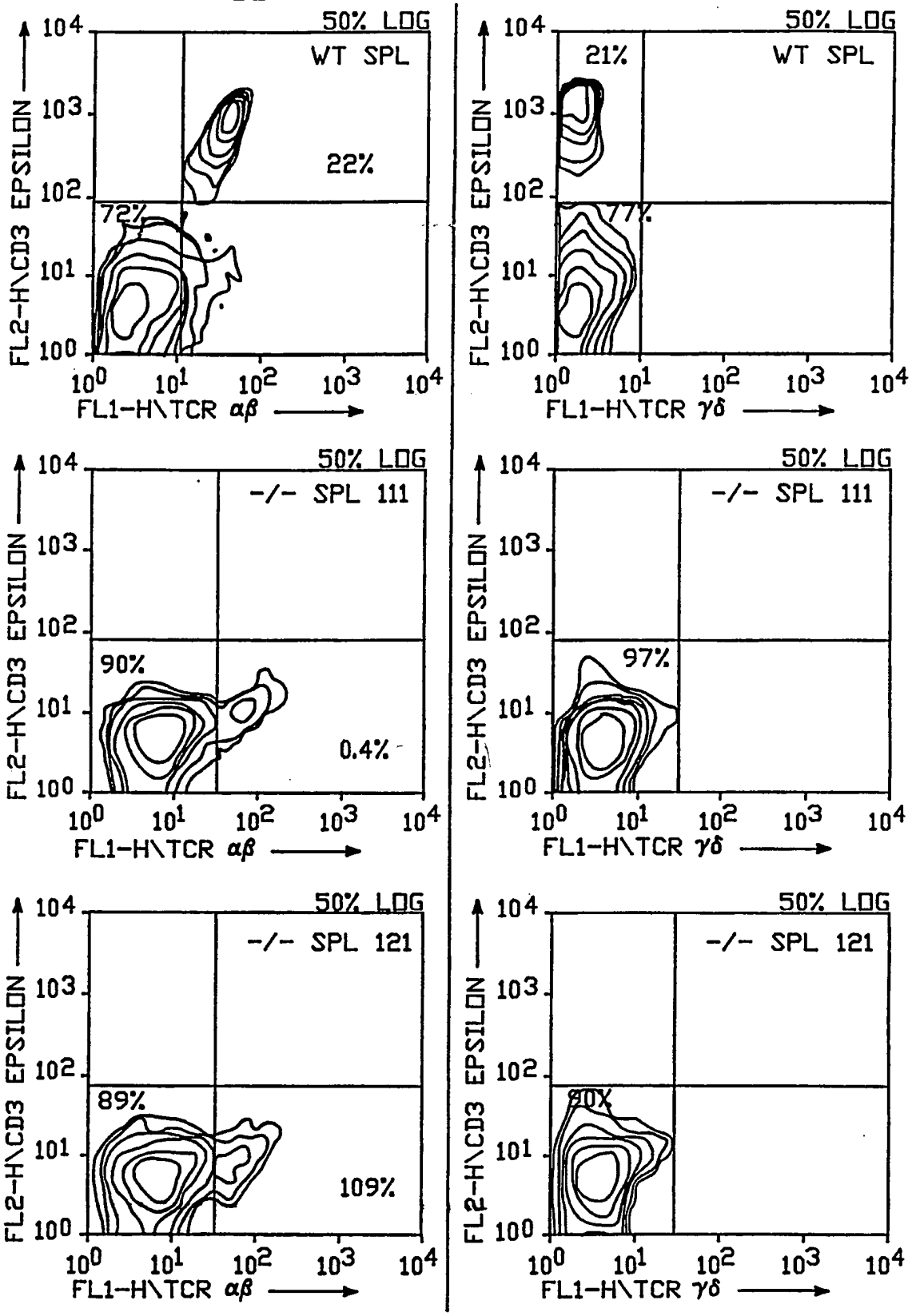

A
B
50% LOG
WT SPL
22%
72%
21%
77%
-/- SPL 111
90%
0.4%
97%
-/- SPL 121
89%
109%
80%
FL2-H\CD3 EPSILON
FL1-H\TCR αβ
FL1-H\TCR γδ
10^0
10^1
10^2
10^3
10^4
100
101
102
103
104

FIG.—19

C

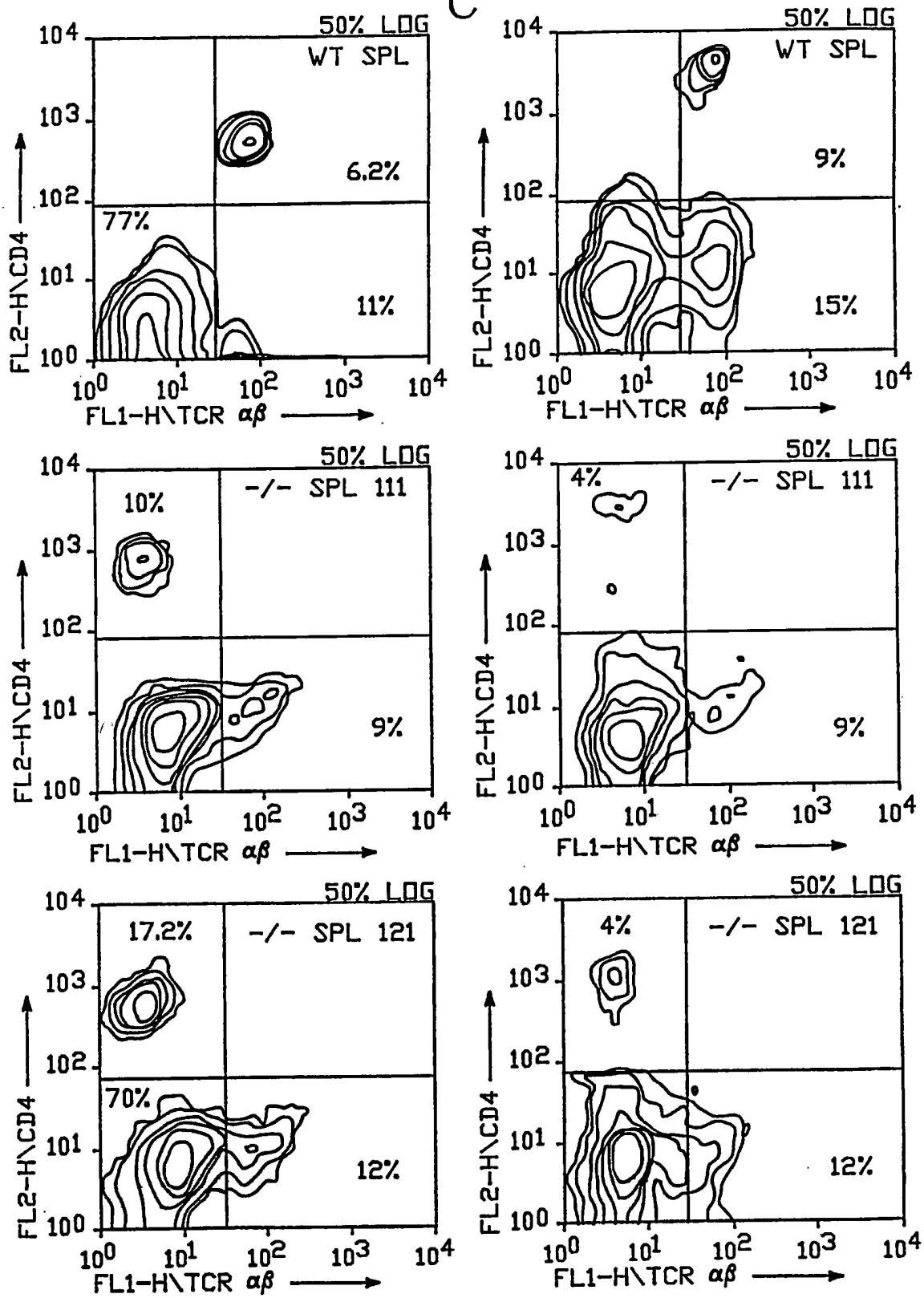

50% LOG
WT SPL
6.2%
77%
11%
9%
15%
-/- SPL 111
10%
9%
4%
-/- SPL 121
17.2%
70%
12%
4%
FL2-H\CD4
FL1-H\TCR αβ
10^0 10^1 10^2 10^3 10^4
100 101 102 103 104

FIG.-20

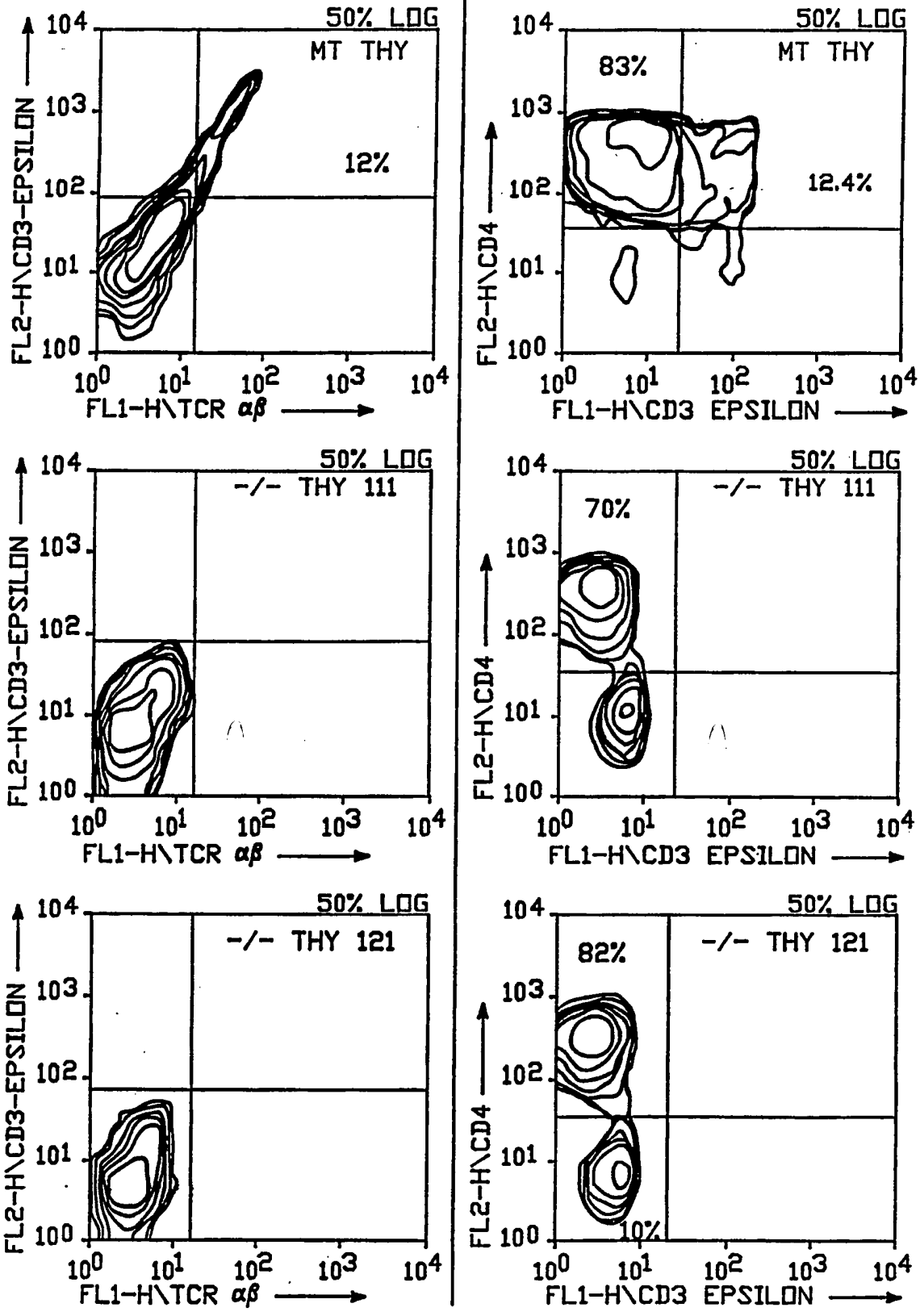
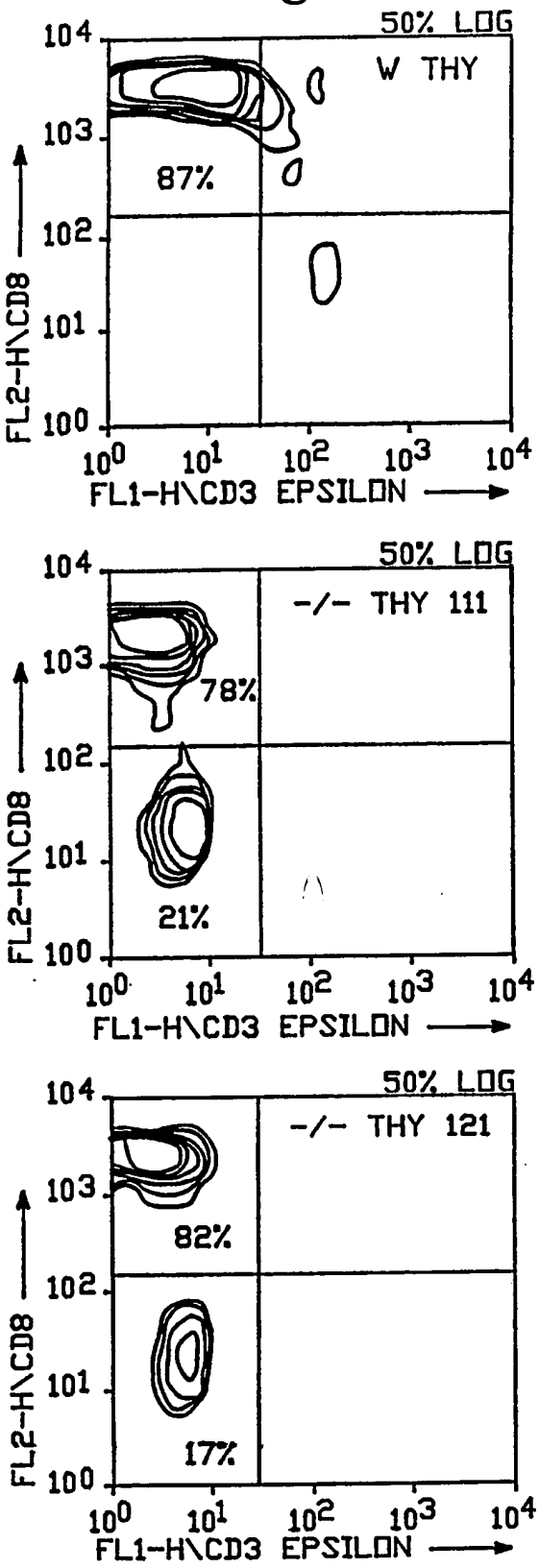

A
B
50% LOG
MT THY
12%
83%
12.4%
-/- THY 111
70%
-/- THY 121
82%
10%
FL2-H\CD3-EPSILON
FL1-H\TCR αβ
FL2-H\CD4
FL1-H\CD3 EPSILON
10^0
10^1
10^2
10^3
10^4
100
101
102
103
104

FIG.—20

C


50% LOG
W THY
87%
50% LOG
-/- THY 111
78%
21%
50% LOG
-/- THY 121
82%
17%
FL2-H\CD8
FL1-H\CD3 EPSILON
10^0
10^1
10^2
10^3
10^4
100
101
102
103
104

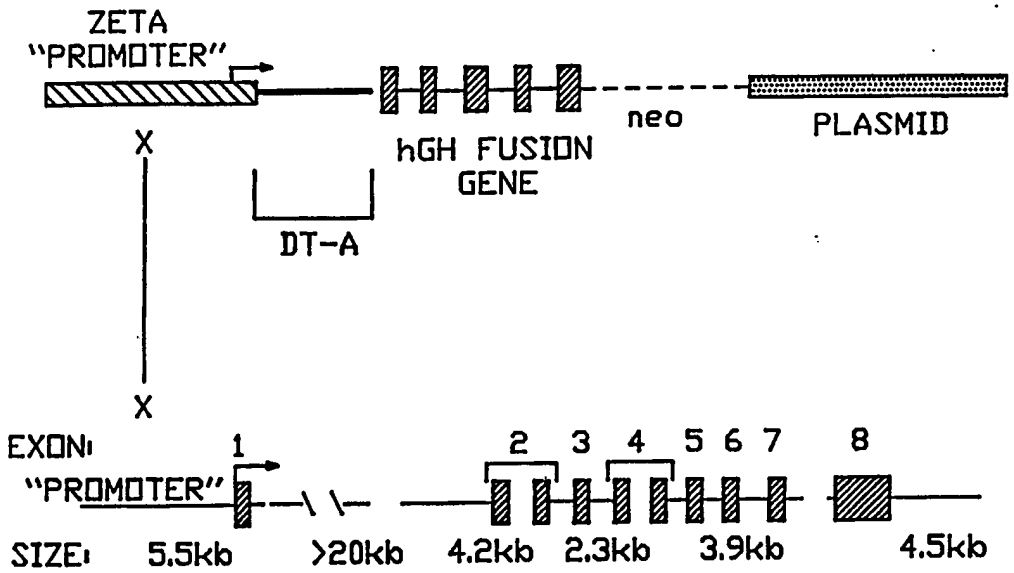


FIG.—21A

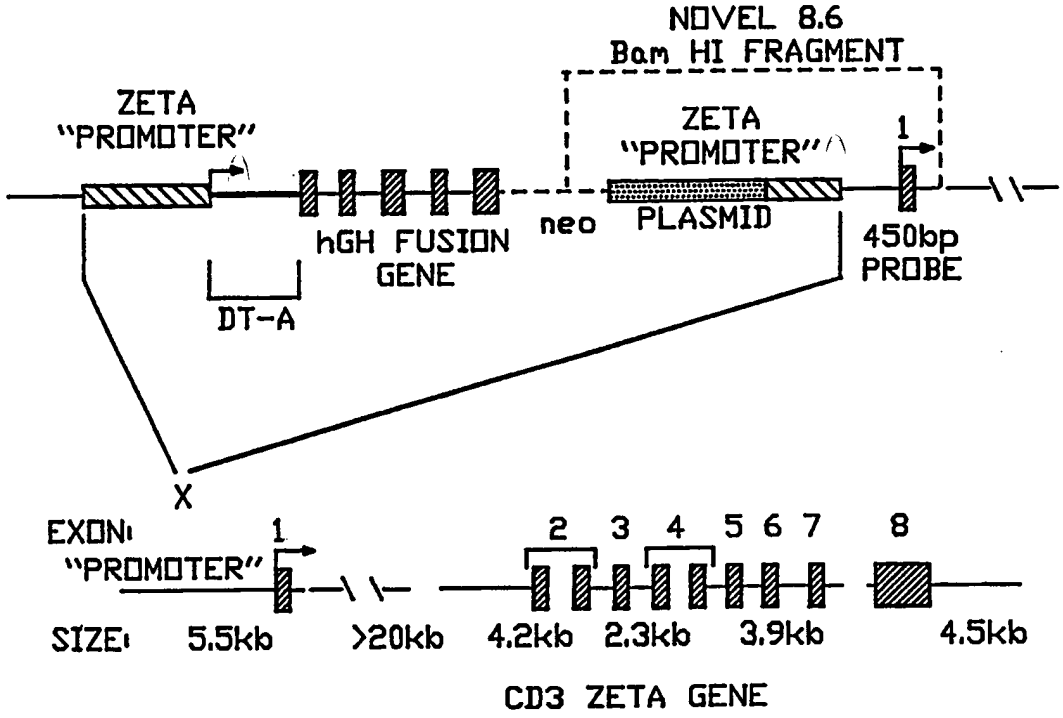


FIG.—21B

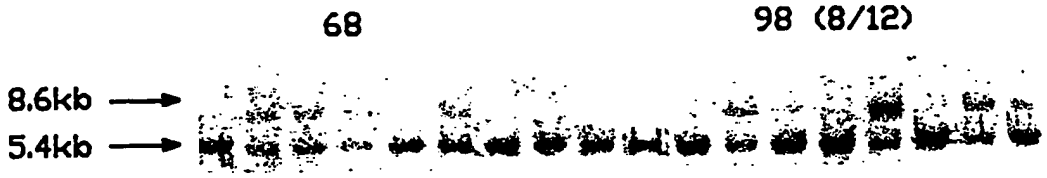


FIG.—21C

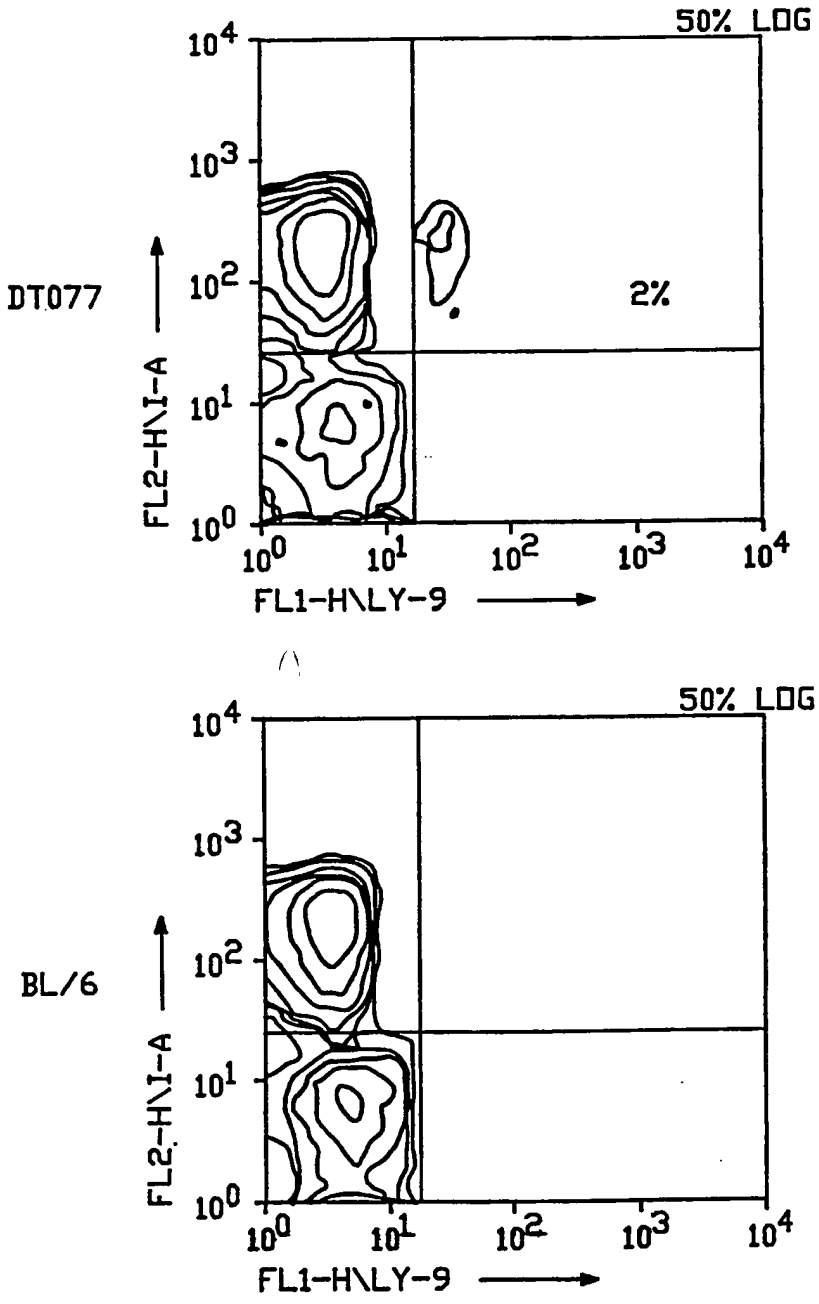
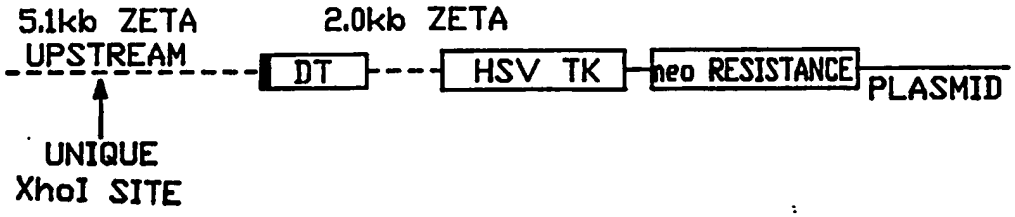
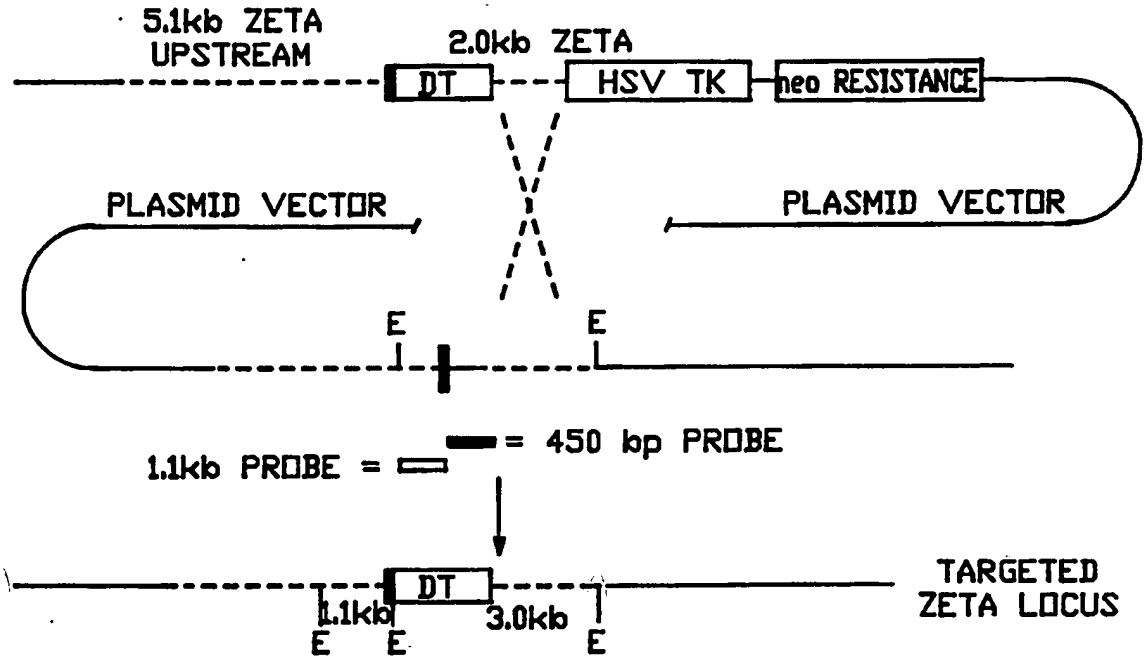


FIG.—22



ZETA/DT HIT AND RUN VECTOR ▮ = UNTRANSLATED REGION OF CD3ζ EXON 1

FIG.—23A



INTRACHROMOSONNAL REVERSION BETWEEN THE 2.0kb DUPLICATED ZETA FRAGMENT AND THE ENDOGENOUS 2.0kb FRAGMENT. DOTTED LINES REPRESENT THE DUPLICATED REGIONS ARISING FROM RECOMBINATION OF THE TARGETING VECTOR. THE DESIRED CROSS-OVER EVENT IS SHOWN THAT LEADS TO A LOOPING OUT OF BOTH SELECTABLE MARKERS AND PLASMID DNA.
(E = EcoRI ▮ = UNTRANSLATED REGION OF CD3ζ EXON 1)

FIG.—23B

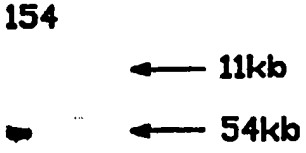


FIG.—23C

I ERNATIONAL SEARCH REPORT

International Application No PCT/US 92/04823

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/00; A01K67/027; C12N15/12; C12N15/31
C12N5/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,9 012 087 (NOVACELL CORPORATION) 18 October 1990 see page 4, line 3 - line 39 see page 7, line 6 - page 8, line 6 see page 22, line 4 - line 18 ---	1,2,6
E	EP,A,0 494 776 (UNIVERSITY OF EDINBURGH) 15 July 1992 see the whole document ---	1,6-8, 16,18, 26,31
A	NATURE. vol. 344, no. 6268, 19 April 1990, LONDON GB pages 742 - 746; ZIJLSTRA, M. ET AL.: 'Beta-2-microglobulin deficient mice lack CD4-8+ cytolytic T cells' see the whole document --- -/---	19,23

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 SEPTEMBER 1992	02. 11. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	SCIENCE. vol. 241, 23 September 1988, LANCASTER, PA US pages 1581 - 1583; YANCOPOULOS, G.D. ET ALT, F.W.: 'Reconstruction of a immune system' see page 1582, column 2 - page 1583 ---	24,25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04823

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 40 is directed to a method of treatment of the animal body the search has been carried out and based on the alleged effects of the compound/composition when we considere the term of xenograft being right instead of xenograph

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9204823 SA 61507

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 14/09/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9012087	18-10-90	EP-A- 0466815	22-01-92
EP-A-0494776	15-07-92	None	